INTER-SITU RESTORATION AND THE USE OF WHOLE SOIL INOCULA TO REHABILITATE THE CRITICALLY ENDANGERED HAWAIIAN PLANT, *CYRTANDRA KAULANTHA*

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ABSTRACT

As native plant habitat continues to degrade, alternative restoration practices need to be methodically evaluated. *Inter-situ* restoration can be used as a platform to identify best practices for rehabilitating endangered plant species in novel ecosystems. In this study, we explore the use of whole soil inoculation in the greenhouse as a low-cost technique for enhancing the survivorship and growth of the Hawaiian species, *Cyrtandra kaulantha*, when outplanted at an *inter-situ* restoration site. Symbioses between terrestrial plants and soil microbiota are common across most genera and are considered beneficial to plant growth, however, such relationships are rarely studied in endangered flora. Thus, we used whole soil inoculation techniques to test these potential benefits. *Cyrtandra kaulantha* individuals were propagated from cuttings and grown in 1 of 5 soil treatments in the greenhouse: sterilized control media; whole soil inoculum from the native reference site; whole soil inoculum from the *inter-situ* restoration site; a phosphate amendment; and a mixed trial of the *inter-situ* soil and phosphate. After 10 weeks in the greenhouse, root samples were collected and stained for mycorrhizal colonization and *Cyrtandra kaulantha* individuals were outplanted. Morphological measurements (i.e., height, stem diameter, leaf area, leaf number) and survivorship data were collected monthly for each individual. Root staining analysis showed evidence of colonization by arbuscular mycorrhizae in individuals grown in the *inter-situ* whole soil inoculum after the greenhouse trial. There were no significant differences in morphological measurements by treatment after the 12-month field trial. Differences in survival rates by treatment approached significance, with both whole soil treatment groups demonstrating trends towards higher survival rates during the field study. Based on these results, it is possible that interactions between whole soil inocula and *Cyrtandra kaulantha* may have impacted initial survivorship and establishment. If supported by further research, whole soil inoculation could be a low cost method to increase the success of restoring rare plant species to novel ecosystems in Hawaiʻi. *Inter-situ* restoration techniques are a valuable way to empirically test best practices for the restoration of endangered species.

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CHAPTER 1. LITERATURE REVIEW

1.1 INTRODUCTION

Human related disturbances and climate change have led to widespread extinction and endangerment of more than 85% of the native Hawaiian flora (Wagner et al. 1999). In attempts to combat this detrimental loss of biodiversity, management groups rely heavily on habitat restoration and reestablishment of wild collected species. However, in Hawaiʻi the success of these practices is largely unquantified and, thus, not adaptable to rapidly changing plant communities and continuously degrading ecosystem functioning (Price and Toonen 2017). Furthermore, due to their endangerment, there is often little empirical information about the habit or historical range of these species. In order to make assessments and provide methodologies for the recovery of endangered flora, translocations and intermediate restoration projects can be established in novel ecosystems to augment struggling populations and create opportunities for more rigorous experimental inquires (Cordell et al. 2016).

Recent research suggests that effective plant restoration may be linked to the composition and availability of soil microbial communities in an area (Zhang et al 2012, Paluch et al. 2013). Thus, being able to understand the relationship between local soil microbial communities and their host species is key to successfully re-establishing native species in altered ecosystems and could increase the success of endangered plant restoration actions in Hawaiʻi (Wubs et al 2016). Introduction of beneficial soil microbiota to seedlings during greenhouse cultivation has been identified as a viable strategy to enhance plant growth and resiliency for certain species

(Dumerose et al. 2015, Idol and Diarra 2017). Our study attempts to identify how the use of whole soil inoculation during propagation impacts the establishment of *Cyrtandra kaulantha* in an *inter-situ* restoration site.

Cyrtandra kaulantha is a critically endangered and federally listed endemic Hawaiian plant species. The species does well in the greenhouse, but has not been successfully re-established in the field to date and without direct human intervention, this species is at high risk of extinction. Our research examines how introduction to local and native soil microbiota during root development might impact the survival and growth rate of the species. If successful, further development of this methodology could greatly impact the viability of this and other native Hawaiian plant species. We use techniques that are easily replicable and feasible within the constraints of local land management in Hawaiʻi in the hopes that our work can be applied directly.

1.2 REVIEW OF ECOLOGICAL RESTORATION

Current State of Restoration Ecology

Globally, ecological restoration is regarded as a necessary tool for reversing the loss of ecosystem structure and function caused by anthropogenic disturbance. A primary objective of these restoration efforts is to reintroduce native species back into altered systems to improve population dynamics (Powell et al. 2017). If correctly implemented, reintroductions can help strengthen genetically vulnerable populations, re-establish resilient ecosystem function, and augment existing populations (Godefroid 2011, Richards et al 2016). Much like natural processes, reclamation and rehabilitiation have the potential to move degraded systems closer to their original state (Dobson et al. 1997).

As rates of disturbance continue to grow, managers will need to rely more heavily on restoration and reintroduction techniques to combat high rates of disappearance (Ammondt et al 2013). Unfortunately, the literature suggests that many projects are often unsuccessful due to methodological gaps and the absence of long-term monitoring to inform best practices (Albrecht et al. 2012, Vitt et al 2016). Many studies fail to identify or remove the underlying cause of decline in current populations, and approaches are not always based on known requirements for success (Godefroid et al. 2010, Price and Toonen 2017). Thus, there is a need for tested and tailored best-practice guidelines prior to implementation. Researchers need to embrace adapative restoration techniques and integration of increasingly common novel ecosystems as circumstances become more dire (Hobbs et al. 2009; Palmer et al 2016). Furthermore, a "one size fits all" approach to restoration is not appropriate for modern ecosystems (Riley et al 2015) and proritization of restoring resiliency may become more important than"historic authenticity". In order to successfully combat habitat loss and reduce extinction risk, restoration practices must become more place- and process-based, and have a foundation of empirical research (Palmer et al. 2016).

Restoration Limitations in Hawai'i

The unique evoluntionary pressures created by Hawai'i's geographic isolation, varied climate, and dramatic topography, has led to some of the highest endemism and speciation rates in the world (Ziegler 2002). Unfortunately, due to their adaptation to narrow ecological ranges, micro-endemic species are often the most susceptible to environmental shifts (Bialic-Murphy et al. 2017). The native flora of the Hawaiian islands is greatly impacted by negative interactions with invasive species, whose presence threatens historic ecological processes. Competition for resources by invasive plant species, predation by introduced mammals and invertebrates, and loss of seed-dispersal mechanisms are amongst the greatest threats to native plant populations (Wilcove et al 1998, Shiels et al. 2011, Hernández-Yáñez et al. 2016).

Although it represents a critical need, restoration in the state of Hawaiʻi is limited by a number of factors including a general lack of financial resources, lack of species specific research on which to base restoration parameters, and a lack of physical accessibility to restoration sites. Furthermore, working with rare plants means that managers are frequently dealing with small samples of highly regulated and relatively unknown species (Krushelnycky et al. 2016). Intense degradation has greatly diminished reference habitats and remaining pristine habitat is often confined to inaccessible peaks, high elevation areas and gulches. Inacessibility creates phyiscal challenges to management agencies and hinders regular monitoring and *in situ* observations. These pressures and gaps in data often force managers to create restoration plans based on general or personal knowledge as opposed to species/ecosystem specific theory (Price and Toonen 2017). The unique limitations on Hawaiian habitat, make outplantings infrequent and thus critical because they are often viewed as a "one shot deal" (Cordell et al 2016). These narrow parameters make it necessary to decrease uncertainty prior to ouplanting actions by understanding the specific factors that impact the success of restoration of each species in Hawaiʻi. Site and species specific experimentation needs to be conducted and adapted to local systems (Godefroid et al 2011, Albrecht et al. 2012, Cordell 2016) to improve chances of ecological restoration.

Translocation and Assisted Colonization

The majority of plant reintroduction efforts to date rely primarily on the use of *ex situ* propagation and storage (i.e., greenhouses, seed banks, micropropagation laboratories) and *in situ* outplanting and restoration. However, as pristine *in situ* habitats continue to decrease in function and become more fragmented, intermediate types of reintroduction and utilization of novel ecosystems is increasingly necessary (Dalrymple 2012; Volis et al. 2016). Although it is widely agreed that *in situ* restoration of species within their native range is ideal, the reality of future unpredictable range shifts and ecosystem degradation due to the effects climate change challenge this framework (Vitt et al. 2016). In order to conserve rare species before extinction, reintroduction of individuals into a variety of different habitats must be considered. Conceptual models can help to determine the appropriate actions for management based on level of risk and likelihood of reduced threat (Fig. 1.1 , Jachowski et al. 2015).

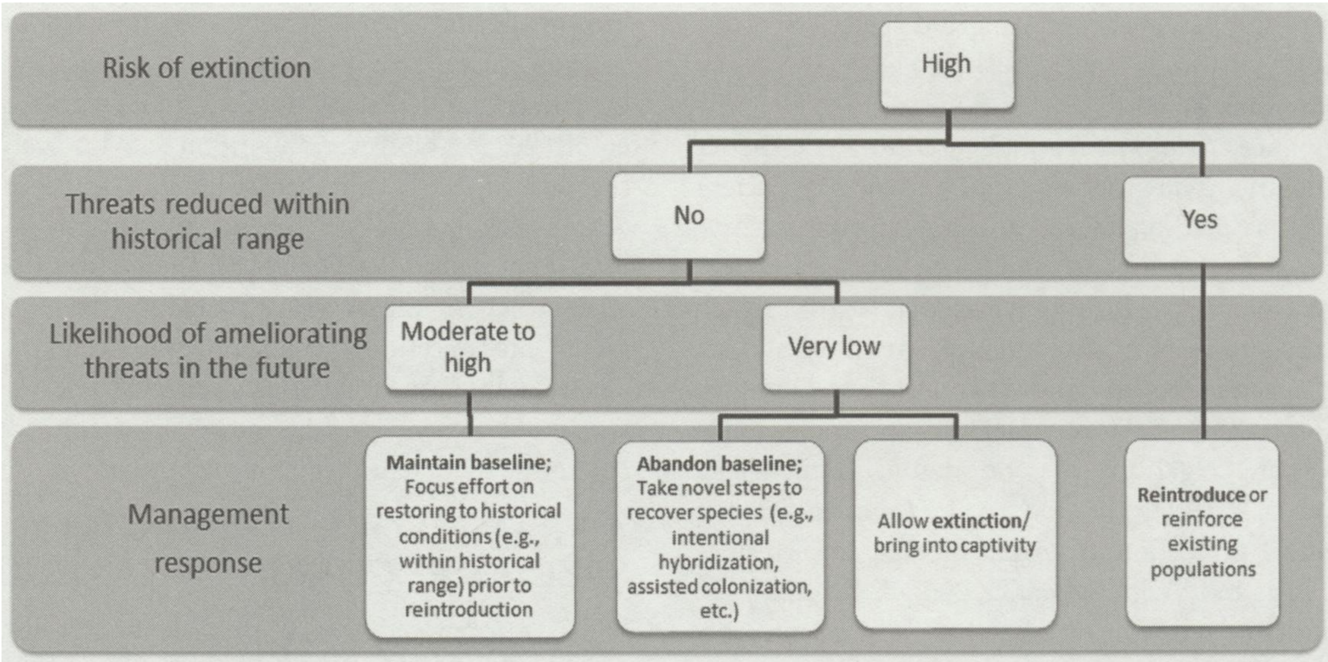

Figure 1.1 Conceptual model for determining the appropriate management response to risk of extinction based on threat type and durations, including novel approaches like assisted colonization (Jachowski et al. 2015)

Assisted colonization is a type of reintroduction that is specifically utilized as an extreme conservation introduction method, by placing species outside of their known indigenous range to prevent extinction (IUCN/SSC 2013; Sandler 2010; Vitt et al 2016). Sensitive species, like the numerous endangered flora of Hawaiʻi, are often isolated by topographic or human-created barriers and will likely become extinct as their reference habitat degrades. These species require habitat with limited predation, invasion, and competition (Hobbs et al. 2006) and with protection from deleterious legacy effects (Maschinski and Qunitana-Ascencio 2016). Assisted colonization of these flora to a similar, but more stable "safe haven" can create opportunities for a dwindling population to recruit and repopulate (Dalrymple 2012). These efforts can ensure the continuation of a population that no longer has a suitable reference habitat while increasing overall ecosystem function, especially for species that have been extirpated from their historical site (Maschinski and Quintana-Ascencion 2016; Yelenik et al. 2017).

Inter-situ Restoration

Similar to assisted colonization, *inter-situ* restoration is a semi integrated restoration practice for rehabilitation of rare species that allows for some site manipulation (Shaw 2019). There are numerous terms used in the literature to describe similar types of reintroduction practices such as "translocation", "managed relocation", or "augmentation" (Vitt et al 2016). For the purposes of this study we will use the term "*inter-situ*" restoration to mean the small-scale introduction of a rare species to a novel ecosystem outside, but adjacent to, its native range for conservation purposes. *Inter-situ* restoration greatly increases accessibility by managers to monitor outplantings and allows for easy implementation of experimental treatments in a field setting (Miller et al 2012). *Inter-situ* restoration has been applied to monitor the impacts of rhizomal pathogens on threatened plant species native to Western Australia (Cochrane et al 2010). The latter study planted native species in an easily accessible, but novel area with limited invasion in order to identify ecosystem interactions and draw conclusions about the plant for later restoration efforts. The use of *inter-situ* restoration allowed researchers to manipulate a separate population of a rare species and learn more about….. prior to enacting a larger restoration effort and thereby decreasing risk and unforseen consequences to the source population. *Inter-situ* restoration also provides a unique opportunity to study recruitment of rare plants in novel ecosystems which will become more common with continued climate change (Braidwood et al 2017). In this study, we will use an *inter-situ* restoration site to evaluate the impacts of soil microbial interactions on the successful reintroduction of an endangered species.

1.3 REVIEW OF PLANT-SOIL MICROBIAL INTERACTIONS

Plant and soil microbiome interactions

Over 70% of terrestrial plants form symbiotic relationships with microorganisms like Arbuscular Mycorhizal Fungi (AMF) and plant growth promoting rhizobacteria (PGPR). These microorganisms are found naturally in most soils and have co-evolved mutualisms with terrestrial flora around the world (Bilj et al 2011; Wurzburger and Clemmensen 2018). AMF and PGPR colonize young plant roots to gain access to carbon pathways created by plant photosynthesis. As a trade off, colonizers facilitate the plant uptake of key nutrients and minerals like Phosphorous and Nitrogen which play critical roles in plant growth and development (Kobae 2016, Flores-Gallegos and Nava-Reyna 2019). These relationships enhance plant fitness in numerous ways including increased plant host tolerance to extreme pH levels and resiliency to climatic variation (Ahmed and Kibret 2013). Studies have also shown that positive symbiotic relationships between soil microbiota and plants can increase host defenses against herbivory, pathogenic distress, and high salinity (Middleton 2015; Ilangumaran and Smith 2017; Koziol et al. 2017). The presence of PGPR's and AMF have been shown to increase the biomass and survivorship of plant species in the field (Kloeppe et al. 1999; Trognitz et al. 2016).

When planning plant translocation efforts it is important to consider the availability and composition of the belowground community and its possible influence on outplanting success. Restoration-based research is increasingly focused on quantifying these ecological interactions and determining best practices for soil analyzation and amendment. As soils become more degraded, enhancement of natural plant uptake will be increasingly important for many species

(Trabelsi and Mhamdi 2013). Relationships between beneficial soil microbiota and flora are an important component of ecosystem function and may play a critical role in rare plant restoration success (Bever et al. 2010). If these relationships are better understood, they can be manipulated or anticipated to better enhance growth of native plants in the field.

Hawaiʻi specific plant-microbiome interactions

High rates of endemism and speciation in the Hawaiian archipelago are ubiquitous across taxa, and soil microbial communities are no exception. Initial analysis of these communities across the Hawaiian islands show differences in the composition of microorganisms dependent on their physical location within the landscape. Recent work by Hynson et al. (2018) suggests that soil microbial communities from topographically isolated valleys within the same mountain range on the island of Oʻahu were composed of vastly different microbial species. This preliminary work demonstrates the potential for great differences in biodiversity based on region. Thus, if native Hawaiian plants have high mutualistic tendencies with co-dispersed soil microbiota, the exact composition of these belowground communities might greatly impact the type and quality of the relationships formed.

To date, it is unclear how specific these relationships are and how susceptible native plants are to changes in the microbial community due to translocations and degradation. To address this gap, research has turned toward assessment of the structure and compsition of microbial communities in Hawaiʻi. A study by Vannette et al. (2016) quantified changes in microbial biodiversity and associated effects in fragmented forests of *Metrosideros polymorpha*. This was one of the first local studies to highlight the impact of habitat connectivity and ecosystem function on specific mycorrhizal symbioses. In 2018, a task force called the Center for Microbiome Analysis through Island Knowledge and Investigation (C-MAIKI) began to address these gaps and identify specific habitats and diversity of microbial communities across ecosystem types in the Hawaiian islands (Hynson et al. 2018). Predictions from this group and other researchers will have important implications for the future of plant endangerment and restoration efforts in the state of Hawaiʻi. If changes in symbionts directly impact plant establishment, restoration and conservation of both below- and above-ground biota will become necessary. Therefore, further exploration of these complex relationships is key to find methods to conserve the rare flora of Hawaiʻi.

Use of mycorrhizal and bacterial inoculation for plant restoration

More than a century ago, agricultural researchers developed a technique to isolate specific species of growth-enhancing mycorrhiza and rhizobacteria and started producing commercial inoculants for use with common agricultural crops (Brown 1918). The species used are often generalist strains of the fungal genus *Glomus* and the bacteria *Rhizobium*; both are chosen due to their ability to create symbioses with a variety of plant hosts. The development of this technique has positive implications for use in native plant restoration, however, the benefits to rare plants is not clear as contemporary ecological research questions the effectiveness of commerical inoculants made from generalist soil microbiota on highly specialized, uncultivated plant species (Ji et al. 2010; Dumroese 2012).

Although soil symbionts generally seek mutualisms with accessible plants, there is a level of host-specificity and host-preference that determines the strength and likelihood of these relationships (van der Heijden et al. 2015; Maltz and Treseder 2015). Among other factors, the actual source of mycorrhiza and rhizobacteria greatly influences the effectiveness of soil inoculation on plant growth, particularly in native plant species. For example, Paluch et al. (2013) compared the effects of inoculum made from native AMF strains and commerical inoculum made from generalist strains on the growth of native plant species in the field and demonstrated that addition of native inoculum produced higher rates of host colonization than the commercial strain. Similarly, a study by Nunez et al. (2009) examined the potential of invasive plant species to be limited by access to regional microbiota from their reference habitat. Absence of symbiotic microbiota from a species' native range can decrease dispersal and recruitment. These, and other studies (Williams et al. 2012, Zhang et al. 2012, Estrada et al. 2013) suggest that native plants interact with different soil symbionts in a variety of ways and that the significance of these interactions is dependent on the origin of each species (Emam 2016). Therefore, inoculants created from soils indigenous to a plant's native range, may be more effective in enhancing plant growth than those created for commercial use becuase of positive co-evolution.

Like all ecosystems, the community composition of each regional microbiome alters the overall functioning of the system. In other words, the unique combination of fungi, bacteria, and other microorganisms in the soil can alter the relationship between plant and microorganism, thus affecting the amount of benefit received (Ji et al. 2010; Trabelsi and Mhamdi 2013). Several factors, including the increase of non-native host plants, changes to soil physical traits and the influx of Nitrogen from anthropogenic sources may alter the composition of microbial communities belowground (Johnson et al. 2013). These changes impact the availability of niche symbionts for native plant species and can thus change the suitability of the historical environment.

Accordingly, understanding the make up of local and reference soils can help inform the use of inoculants in restoration and translocation practices. For example, as fungal communities shift in response to increased availability of Nitrogen, there will be fewer microbiotic species that facilitate phosphorous uptake (Maltz and Treseder 2017). Phosphorous is a key nutrient for plant growth and root development but is easily depleted in the rhizosphere due to low soil mobility (Kobae et al. 2016) . Without the presence of appropriate beneficial mycorrhizae, threatened plants species may further decline and reintroductions will be less successful (Bolduc 2011). As such, the use of soil inoculation from native reference sites should be considered when translocating rare species in order to bolster resiliency in changing ecosystems.

Inoculation Techniques

There are several techniques for producing inoculants from native or local soils, each of which have their own benefits and shortcomings (Table 1; Koziol et al. 2017). One of the most well researched techniques is mycorrhizal culturing. In this method, specific AMF strains are isolated in a laboratory setting and processed into a non-pathogenic liquid. Although reliable, this method is time-intensive, and requires expensive laboratory equipment and sterile growth conditions. It also excludes any benefits that might be gained from the presence of other beneficial microbes like PGPRs (Koziol et al. 2017). This method is appropriate in many circumstances, but is not necessarily realistic for use by managers with limited time and funding.

Conversely, whole soil inoculation involves the use of soils collected from the rhizosphere as unaltered amendments that are directly added to a growth media. This process is cheaper, more inclusive, and more accessible to practitioners who may lack expertise and laboratory space (Paluch 2013). Furthermore, Emam (2016) suggest that creating a whole soil inoculum may provide a broader array of soil microbes, not limited to AMF. A collection of topsoil including the rhizosphere, allows for interactions between all microbiota and possibly facilitates beneficial symbioses we are currently unaware of (Requena et al. 2001, Ji et al. 2010). Use of whole soil inoculum does have the potential to introduce pathogens to plants in the greenhouse, however, this risk can be minimized with a thorough drying of the soil. Whole soil inoculation has the potential to postively impact outplanting success of native Hawaiian plant species commonly grown in soils with high ambient levels of fungal spores, but has not been extensively tested. If successful, this technique could be utilized for quick actions necessary for conserving species that are “on the brink”.

Table 1.1 Comparisions of the different types of microbial inoculum (from Koziol et al. 2017).

Types of Microbial Inoculum

Whole Soil Inocula from Reference Ecosystems	Trap Cultured Microbes	Mycorrhizal Cultures
PROS: • Contains the complete array of the soil community including AM fungi, beneficial bacteria pathogens, soil-dwelling insects, nematodes, and even seeds or other plant propagules. • Easiest way to find locally adapted, reference ecosystem microbes with the lowest associated cost.	**PROS:** • The trap culture method is a way to increase a small volume of whole soil inocula into a larger volume of whole soil inocula. This results in less disturbance of reference ecosystem soils. • Early iterations of trap cultures contain the complete array of the soil community including AM fungi, beneficial bacteria, pathogens, soil-dwelling insects, and other soil biota.	**PROS:** • Contain beneficial mycorrhizae without harmful plant pathogens. • Many types of commercial mycorrhizae can be easily purchased from a garden store or online. • Recent studies suggest that reference ecosystem mycorrhizae alone may be as beneficial as whole soil inocula, which may reduce the need to disturb remnant grassland soils.
CONS: • Collecting whole soils is destructive to remnant habitats which may be sparse. • Large volumes of whole soil inocula may be difficult or impossible to obtain. • Whole soil inocula may contain pathogens that are harmful to plants. • Effective inoculation rates reported in the literature range from 150-10,000 gallon per acre and lower rates have not been tested.	**CONS:** • Require sterile growing environments and greenhouse or equivalent to avoid contaminating the microbes. • Trap cultures of microbial communities may change over time and it is difficult to determine what is being cultured. For example, microbial species decline or the culturing of weedy species is possible using trap cultures.	**CONS:** • Require training and sterile growing environments to culture fungi or funds to purchase products. • Commercial mycorrhizae that are not locally adapted to a restored community type or location may be ineffective. • The suggested inoculation rates of commercial inocula are largely ineffective and successful inoculation rates have not yet been tested.

Impact of ex-situ *cultivation methods on outplanting success in Hawaiʻi*

Many endangered Hawaiian plants are able to survive in greenhouses, but cannot successfully grow or recruit in the wild once outplanted. In the greenhouse, plants are fertilized, watered regularly and treated with pesticides, and exist in a relatively stable and uniform environment. However, treatment with systemic fungicides and fertilization with Phosphorous greater than 150ppm greatly decreases the likelihood that a plant will form symbioses with AMF and other plant growth promoting microbes (Dumroese 2012). With easy access to nutrients in the greenhouse, plants need not rely on mutualist relationships to enhance uptake, however, if such relationships are not established prior to outplanting, greenhouse cultivated plants will likely have more difficulty acclimating to the harsher, more nutrient poor conditions in the field (Kobae et al. 2016). Currently, it is unknown to what degree outplanting failure is due to the changes in environmental conditions, alteration to field sites or shortcomings in greenhouse practices (Price and Toonen 2017), thus, newer research is exploring these connections.

Over the past few decades, local studies have identified several species-specific microbial relationships in sterile greenhouse conditions. A study by Gemma et al. (2002) positively pinponted the "mycorrhizal dependency" of four endemic native Hawaiian species via experimentation with AMF inoculation in the greenhouse. Because Hawaiian flora species are often endangered, there is limited to no documentation on which species have mycorrhizal associations. Similarly, Habte et al. (2011) tested the ability of AMF inoculuation to increase the resistance of *Leucaena leucocephala* to extreme pH levels found in Hawaiian ultisols by growing plants in local, sterilized soils (the invasive non-native species was chosen due to limited permitting restrictions). This is one of several Hawaiʻi-based studies that preliminarily suggest the advantages of applying AMF inoculum to promote growth in tropical host species, especially native species. These studies identify the exact interactions between AMF and their hosts in a controlled setting, however, these successes do not always translate to application in a field-based setting.

Continued survivorship and recruitment after outplanting is the primary goal of endangered species restoration. Therefore, it is necessary for the efficacy of greenhouse practices to be tested against successful outplanting in the field. In order to determine if greenhouse inoculation methods can be applied more broadly to ecological restoration practices, researchers in Hawaiʻi have started to explore effectiveness for each individual species. A recent study by Zahn and Amend (2017) tested the use of foliar ectomycorrizal sprays on the critically endangered native Hawaiian species *Phylostegia kaalaensis* as a tool to promote disease resistance and limit fungicide use. Previously unable to survive outside of the greenhouse, *P. kalaaensis* individuals treated with this spray during cultivation now make up the entire reintroduced extant population in the world. The authors use their findings to demonstrate the need for "low-tech" microbial treatments that can be applied in the greenhouse to help boost host species after outplanting. Another local study tested the relationship between *Acacia koa* seedlings grown in different fertilizer levels and under AMF inoculation treatments (Idol and Diarra 2017). Their results are noteworthy because it follows the impacts of cultivation treatments from the greenhouse into a field study environment. Both studies highlight the importance of understanding the complex relationships between plants and microbiomes and from greenhouse to outplanting in the field.

1.4. PRELIMINARY STUDY

A preliminary study for this project was developed by managers at the Hawai‘i Plant Extinction Prevention Program (PEPP) and their partners at the Hawai‘i Rare Plant Program (HRPP) by introducing a critically engandered native plant species, *Cyrtandra kaulantha*, into an *inter-situ* restoration site. This exploratory project was established on the grounds of Harold L. Lyon Arboretum in Mānoa Valley (hereafter the PEPP *inter-situ* site) in October, 2016. *Cyrtandra kaulantha* (*Cyr kau*) is native to the Waiahole area of Waikāne Valley and is a vegetative, gulch-dwelling species. *Cyr kau* was determined to be an appropriate species for translocation because it fits the critically endangered status, has a highly degraded reference habitat, and poses a low risk for invasion (Stone 2010). The *inter-situ* site location was chosen by project managers because it reflects the observed reference habitat where the extant population exists (i.e., steep slopes, tallus soils, 250-350m elevation, mesic forest, and riparian ecosystem). The overall intention was to plant the target species in a similar environment, in the same mountain range and that was easily accessible to management staff so that plants could be easily monitored.

We planted 40 *Cyrtandra kaulantha* individuals across the *inter-situ* site along the NW bank of ‘Aihualama stream with plant spacing ranging from 1-4 m apart (Appendix A.1, Fig.A.1). Prior to outplanting, we removed all small non-native plants, but left a non-native tree canopy of *Ficus* sp. Survivorship was recorded every month and plants were kept free from weeds ~1m from the base of the plant. None of the habitat conditions were quantitatively measured and there was no plan for follow-up maintenance or experimentation. After one year, 17 individuals remained (survival rate = 42.5%, Fig. 1.2), but most showed limited growth and evidence of pest damage by the Twig borer (*Xylosandrus* sp.) (Appendix A, Table A.1). As of 2019, there were no *Cyr kau* individuals remaining at the site, indicating how problematic such outplantings can be and underscoring the need for better procedures.

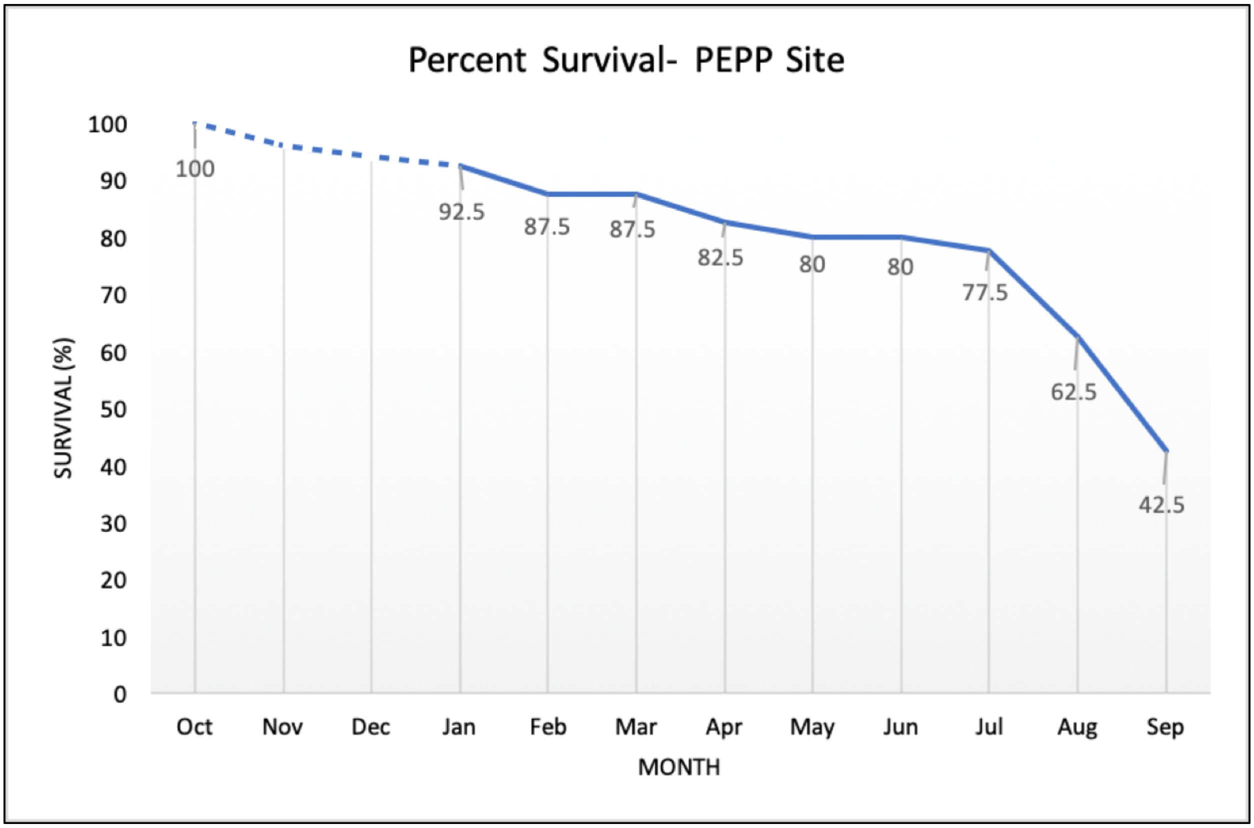

Figure 1.2 Percent survival of 40 *Cyrtandra kaulantha* individuals over a 12 month period at an *inter-situ* site established by PEPP and HRPP on the grounds of Harold L. Lyon Arboretum. The months of November and December are represented as dashed lines because there was no monitoring done during those months.

After 12 months, we took soil samples at the reference site in Waikāne Valley (current population of ~12 individuals) and the PEPP *inter-situ* site previously established at Lyon Arboretum to look for differences that may have impacted outplanting success. Soil testing was done using a composite surface soil sample (0-15 cm depth) and analyzed for pH level, nutrient content of Phosphorous, Calcium, Magnesium, Potassium (ppm), Total Carbon and Total Nitrogen by the Agricultural Diagnostic Center at UH Mānoa (Table 1.2). Comparison of the sites showed lower pH, somewhat lower bases and much lower Phosphorous at the PEPP *inter-situ* site. The generalized adequate soil test for “heavy” Hawaiian soils, suggest normal rates for both PEPP and Waikāne soil samples except for PEPP site which shows lower than normal phosphorous and pH levels (Yost and Uchida 2000). The generalized adequate soil test is generally used for agricultural crops and is not specific to the needs of *Cyrtandra kaulantha,* however, these levels are likely similarly appropriate criteria for native species.

Table 1.2 Comparison of laboratory nutrient and pH analysis of composite soil samples from the PEPP *inter-situ* site, the Waikāne Valley reference site, and generalized adequate soil test levels for Hawaiian soils (Yost and Uchida 2000) as reference.

		ppm, ug/g	ppm, ug/g	ppm, ug/g	ppm, ug/g	%	%
DESCRIPTION	pH	P	K	Ca	Mg	N	TC
Reference Site- Waikāne Valley	6.2	30	771	4475	1469	0.5	7.1
PEPP Site- Mānoa Valley	5.2	8.5	256	1791	823	0.34	5.3
Generalized Adequate Soil Test Levels for Hawaiian Soils (Heavy Soil)	5.8 -6.6	25-35	200-300	1500-2000	300-400		

This preliminary data helped to direct formal research questions for the subsequent study. The extremely depleted levels of phosphorous in the soils at the PEPP *inter-situ* site (Phosphorous=8.5 ppm/ug/g) could be low enough to inhibit plant growth and survivorship, as phosphorous is a critical nutrient for plant development and root growth. Although a more nutrient rich site may be more preferable, it is important to find solutions to nutrient limitations in rapidly degrading ecosystems. If *Cyrtandra kaulantha* is highly dependent on microbial symbioses for nutrient uptake, it may be necessary for the species to receive inoculation from reference soils to help it cope with the stress of translocation to a new region.

1.5 REVIEW OF LITERATURE ON *CYRTANDRA KAULANTHA*

Cyrtandra kaulantha (locally known as Haʻiwale) is a perennial shrub in the African-Violet family (Gesneriaceae). The species is endemic to the eastern Koʻolau Mountains of Oʻahu and the last known wild populations are in Waikāne Valley on the windward, NE side of the island. *Cyrtandra kaulantha* is found in the dense to medium shade of moist wooded gulches at elevations between 250 and 350m, in wet lowland and cliff ecosystems (Department of Interior 2011, Wagner et al. 1999). The last natural population grow on Waikāne silty clay soils (Soil Survey Staff, Natural Resources Conservation Service) in an area with high average rainfall of just under 4000 m/yr (Frazier et al. 2018).

The Hawaiian *Cyrtandra* genus is prone to hybridization, making it difficult to tell similar species apart. However, *Cyrtandra kaulantha* is recognizable for its tubular white flowers and green fruits that form at the base of the plant's stem. This basal cauliflorous habit is characteristic to *C. kaulantha* and *C. stupantha* (St. John 1966, p. 10, Fig 1.3). *Cyrtandra kaulantha* has extended leaves, winged petioles and exhibits dusty, red new growth at the apical meristem and forms adventitious roots at the base of the stem when growing on cliff walls (Fig. 1.4, Ruisi-Besares, pers.obs). The native pollinator of this species is not documented, but the tubular white flowers are suggestive of moth pollination. Seeds of many pacific *Cyrtandra* species, including the close relative *Cyrtandra dentata*, are passively transported by water and actively dispersed by frugivorous birds (Kiehn 2001, Bialic-Murphy et al. 2017).

In 2003, the IUCN Red List of Endangered Species categorized *Cyrtandra kaulantha* as "Critically Endangered" and it is a Plant Extinction Prevention Program (PEPP) target species of concern. The major threats to the species include competition with invasive plant species, feral pig damage, drought, invertebrate predation and encroachment by human activity (Bruegmann and Caraway 2003). A more recent report from the US Fish and Wildlife Service deemed *Cyrtandra kaulantha* federally endangered, citing a total of 28 wild plants and 12 outplanted individuals (Department of Interior 2011).

Current information from managers at the Department of Forestry and Wildlife (personal communication with Susan Ching and Doug Okamoto 2018) reveals that restoration of this species has been largely unsuccessful. Though easy to propagate and care for in the greenhouse, most outplanting efforts have had low survivorship and no natural *in situ* recruitment. Aside from the remaining plants at Lyon Arboretum's PEPP site, there is only one verified extant wild population and one unverified population on private property in the Koʻolau Mountains. The reference site that *Cyrtandra kaulantha* was wild collected from is extremely degraded and there is no current intention of repopulating this area with additional individuals. Located in the back of a crumbling gulch, the extant population is not a good candidate for *in-situ* restoration.

There is little to no published information about this species and therefore management practices are based on the personal knowledge of people who have worked with the species. These practices are not often based on empirical data or studies. The Pāhole Rare Plants

Greenhouse horticulturist, Doug Okamoto (2018) has never tried to use any AMF or soil microbial inocula on this species so it is uncertain if it will form symbiotic relationships, however, another native Hawaiian *Cyrtandra* species has shown AMF nodule formation. A fact sheet created by the Department of Land and Natural Resources (2013) defined two of the top priorities for this species as a need to create new populations to augment the extant population at the reference site, and the need to determine best practices for horticultural uses to aid in pest management and survivorship.

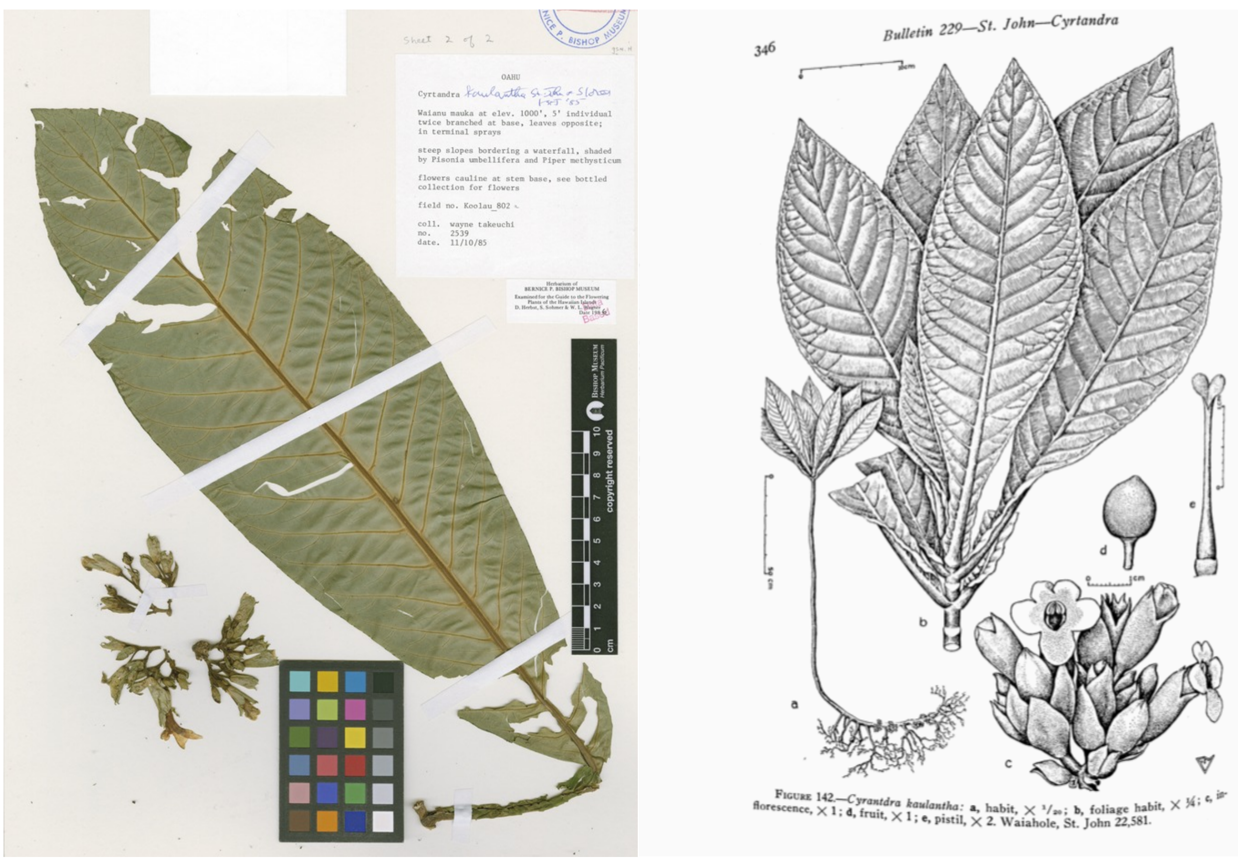

Figure 1.3. (A)Voucher of leaves and flowers of *Cyrtandra kaulantha* from Herbarium Pacificum at the Bishop Museum; collected from Waiahole in 1985. (B) Botanical drawing of physiological characteristics of *Cyrtandra kaulantha* as detailed by St. John in his monograph of Hawaiian Flora (1966).

Figure 1.4 Photographs of the winged petiole (above) and the califlourous, basal forming flowers (below) of *Cyrtandra kaulantha.*

CHAPTER 2: OBJECTIVES AND HYPOTHESES

2.1 OBJECTIVES

The objectives of this study are two-fold:

Objective One:

To evaluate the efficacy of *inter-situ* reintroduction of endangered plant species that have degraded or limited reference habitats, via experimental design and research methodologies.

Objective Two:

To evaluate whether introducing native and local soil inocula during greenhouse propagation increases the growth and survivorship of the endangered Hawaiian *Cyrtandra kaulantha* after it is outplanted in the field.

In order to test the effects of soil inoculation on *Cyrtandra kaulantha*, we propagated individuals in 5 different treatment groups: A control treatment of standard media, a phosphate treatment amended with rock phosphate, a treatment amended with whole soil inocula from the ʻAihualama *inter-situ* site, a treatment amended with whole soil inocula from the Waikāne reference site, and a mixed treatment amended with rock phosphate and whole soil inocula from the ʻAihualama *inter-situ* site. The phosphate treatment is meant to represent the conventional fertilization methods, and the mixed effect treatment is intended to address potential interactions between whole soil inocula and phosphate amendment.

2.2 HYPOTHESES

Main Hypothesis: If there is an effect of whole soil inoculation on plant growth of *Cyrtandra kaulantha*, individuals grown in whole soil treatments will have higher absolute growth and survival rates than those grown in conventional or control treatments.

2.2.1 Greenhouse Study Hypotheses:

H_1: At the end the greenhouse study, *Cyrtandra kaulantha* individuals grown in the Phosphate Amendment (P) and the ‘Aihualama + Phosphate Mixed-Effects (AP) treatments will have significantly higher growth than individuals the other treatment groups (Waikāne Whole Soil (W), ‘Aihualama Whole Soil (A), and Control (C)) across all morphological variables.

H_1 Justification: We expect that during greenhouse cultivation, individuals that receive additional phosphate fertilization will experience higher initial growth. Individuals without it, will grow more slowly.

H_2: During the greenhouse study period, there will be no significant differences in the survival rate of *Cyrtandra kaulantha* individuals between any of the treatment groups (P, AP, W, A, and C).

H_2 Justification: Because the greenhouse is a relatively controlled environment with regular watering and light conditions, we expect low mortality across all treatments. Some transplant shock is expected.

2.2.2 Field (Inter-situ) study hypotheses:

H_1**:** At the end of the 12-month field study, *Cyrtandra kaulantha* individuals grown under the ‘Aihualama Whole Soil treatment and the Waikāne Whole Soil treatment will have significantly higher growth than individuals grown in any of the other treatments groups (AP, P, or C) across all morphological variables.

H_1 Justification: Phosphorous fertilization increases initial growth in the greenhouse but inhibits the formation of beneficial relationships with soil microbiota like arbuscular mycorrhiza (AMF) or Plant Growth Promoting Bacteria (PGRB). *Cyrtandra kaulantha* individuals that were grown in whole soil inocula when young roots were developing will have a higher probability of having formed these beneficial relationships *ex situ* and therefore will better uptake nutrients in the field where phosphorous is limited. Plants exposed to phosphate in the greenhouse will not have formed these relationships and will struggle under harsh field conditions.

H_2: At the end of the field study, *Cyrtandra kaulantha* individuals grown under the Waikāne Whole Soil treatment and the ‘Aihualama Whole Soil treatment will have significantly higher survival than individuals in any of the other treatments (AP, P, or C).

H_2 Justification: *Cyrtandra kaulantha* individuals exposed to whole soil inocula when developing young roots may more easily uptake nutrients, water, and resist pathogens/pests because of their relationships with microbiota like AMF and PGPR that were created during critical root development in the greenhouse.

CHAPTER 3. MATERIALS AND METHODS

This study includes both greenhouse and field experiment components. Any differential growth of *Cyrtandra kaulantha* due to treatments will be measured in the greenhouse. The same individuals will then be tested for treatment impacts on survivorship and growth in field outplantings.

3.1 GREENHOUSE STUDY MATERIALS AND METHODS:

3.1.1 Plant Material and Propagation

Seventy-two *Cyrtandra kaulantha* (*Cyr kau)* individuals were acquired from the Hawaiʻi State Division of Forestry and Wildlife (DOFAW) Rare Plants Greenhouse at Pāhole, Oʻahu. All individuals were offspring of mature plants that were vegetatively propagated from a small population of wild collected plants in Waikāne Valley (Koʻolau Mountains). All individuals were grown in standard potting mix; 10 of 72 individuals were too small and were excluded from the study.

In order to begin our experiment with newly rooted plants, we propagated *C. kaulantha* by making vegetative cuttings, one from each individual plant obtained from Pāhole, then rooted them out in sterile perlite media under mist bench conditions at the Harold L. Lyon Arboretum Greenhouse. Methods of propagation for this species were followed as described in *Growing Hawaiʻi's Native Plants* (Lilleeng-Rosenberger 2016). After sufficient root development (~2 months), cuttings were transplanted into 6" pots with a sterilized standard potting mix (3 perlite: 3 potting mix: 1 coir: 1 cinder) and supplemental amendments were added as prescribed by the experimental treatment. Potting media was sterilized by oven-drying at 40°C for 48 hours (Trevors 1996, Koziol et al 2017, Sawada et. al 2019).

3.1.2 Whole Soil Collection

Soil for inoculation was collected from two sites: the Waikāne reference site where the last known extant population of *Cyr kau* is found and the ‘Aihualama side of Mānoa Valley, where *Cyr kau* cuttings were to be outplanted during the field experiment. Soil was collected as a composite of samples (11.4 liters per site) taken at the rhizosphere (including roots) across the site at a depth of 0.4 m. After collection, soil was air dried for 24-48 hours and chopped up to an even consistency. Large leaves and organic matter were removed by hand and all visible insects were extirpated via the drying process. Collected soils were then used as amendments in whole soil treatments, homogeneously mixed in at a 3:1 ratio of sterilized potting media to whole soil.

3.1.3 Experimental Design

Propagated cuttings of *Cyrtandra kaulantha* were grown under 5 different media treatments in 1 Liter of media:

Control: Standardized potting mix (1L) with no amendments (n=9)

Phosphate: Standardized potting mix (1L) amended with 2oz/gal of rock phosphate(n=9)

Waikāne: Standardized potting mix (0.66L) amended with whole soil collected (0.33L) from the Waikāne reference site (n=10)

‘Aihualama: Standardized potting mix (0.66L) amended with whole soil collected (0.33L) from ‘Aihualama site (n=10)

‘Aihualama + Phosphate: Standardized potting mix (0.66L) amended with whole soil (0.33L) collected from the ‘Aihualama site and 2oz./ gallon of rock phosphate (n=9)

Treatments had 9-10 replicates each (due to mortality during propagation) and were grown under standard greenhouse conditions. Each individual was randomly arranged in the greenhouse via a completely randomized experimental design and monitored for 10 weeks prior to outplanting.

3.1.4 Greenhouse Data Collection

For the 2-mo. duration of this phase of the project, we assessed survival and measured a variety of morphological characteristics to determine growth of each individual plant every 2 weeks. Measurements included; stem diameter, plant height, leaf number and leaf area. Stem diameter was measured by caliper at the lip of the pot (to maintain a consistent height) and was measured to the nearest 0.01mm. Plant height was taken from the soil surface to the bottom of the highest leaf node and measured to the nearest 0.01mm. Leaf area was quantified using the photograph analysis software ImageJ 1.x (Schneider et al. 2012). This method was chosen as a non-destructive measure of biomass *in lieu* of destroying specimens of a critically endangered species. By recording these measurements at the onset and throughout the experiment, we were also able to calculate the Relative Growth Rate (RGR) of each individual and account for differences in leaf area across individuals.

3.1.5 Arbuscular Mycorrhizal fungi- Post Greenhouse Root Staining

Prior to out-planting, root samples were taken from each individual and stained for visualization of arbuscular mycorrhizae. An acid fuschin staining methodology was adapted from “Arbuscular mycorrhizal fungi (AMF) staining protocol” in the Soil Microbial Ecology Laboratory at the University of Hawai‘i- Mānoa (Nguyen and Schechter 2019). Ten cuttings of the youngest roots of each individual (across all 5 treatments) were collected evenly across the surface of the growth medium. After staining, each root was viewed with microscopy techniques to determine the presence or absence of arbuscular mycorrhizal vesicles in the tissue. Observations and photographs were also taken of possible root endophytes (roots were not collected after the field study because our federal permits did not allow for any destruction of the plant in the field).

3.2 FIELD STUDY MATERIALS AND METHODS:

3.2.1 Study Site

The ‘Aihualama experimental site is located on the property of Harold L. Lyon Arboretum (21.33° N, 157.80° W) in Mānoa Valley on the leeward side of the Ko‘olau Mountain range. The outplanting site was located ~450m SWW of the preliminary study site (see section 1.4, Fig. 3.2) on the bank of a rocky gulch in mesic lowland rainforest at ~230m elevation. The site was chosen due to its similarity to the conditions found at the Waikāne reference site including; low-medium canopy cover, minimal ground cover species, well-drained soils, similar precipitation levels, and comparable average seasonal temperature range. The site is located on Tantalus silty clay loam soil (Web Soil Survey, Natural Resources Conservation Service 2019) and had a mean annual rainfall of 3836.6 mm in 2018 (Frazier et al. 2019). This is similar to the rainfall levels at the Waikāne reference site, which had a mean annual rainfall of 3989.4 mm in 2018 (Frazier et al. 2019). The Waikāne reference site is located on the NE side of the island, while the ‘Aihualama *inter-situ* is located roughly 18 km away on the SW side of the island creating potential variation in daily light regime (Fig 3.1).

The site is completely non-native in floral composition with a community predominantly made up of *Cordyline fruticosa, Heliconia* spp.*,* several species of Arecaceae, and *Ardisia elliptica.* There are several larger canopy trees that contribute a significant amount of leaf litter to the understory. Soil analysis of the nearby preliminary study site showed adequate levels of all nutrients except Phosphorus, which was well below the optimal level for good plant growth on volcanic tropical soils (Table 1.2)

Figure 3.1 Satellite image from Google Earth of the relative locations of the Waikane Reference Site and the 'Aihualama *Inter-situ* site and the distance between the two.

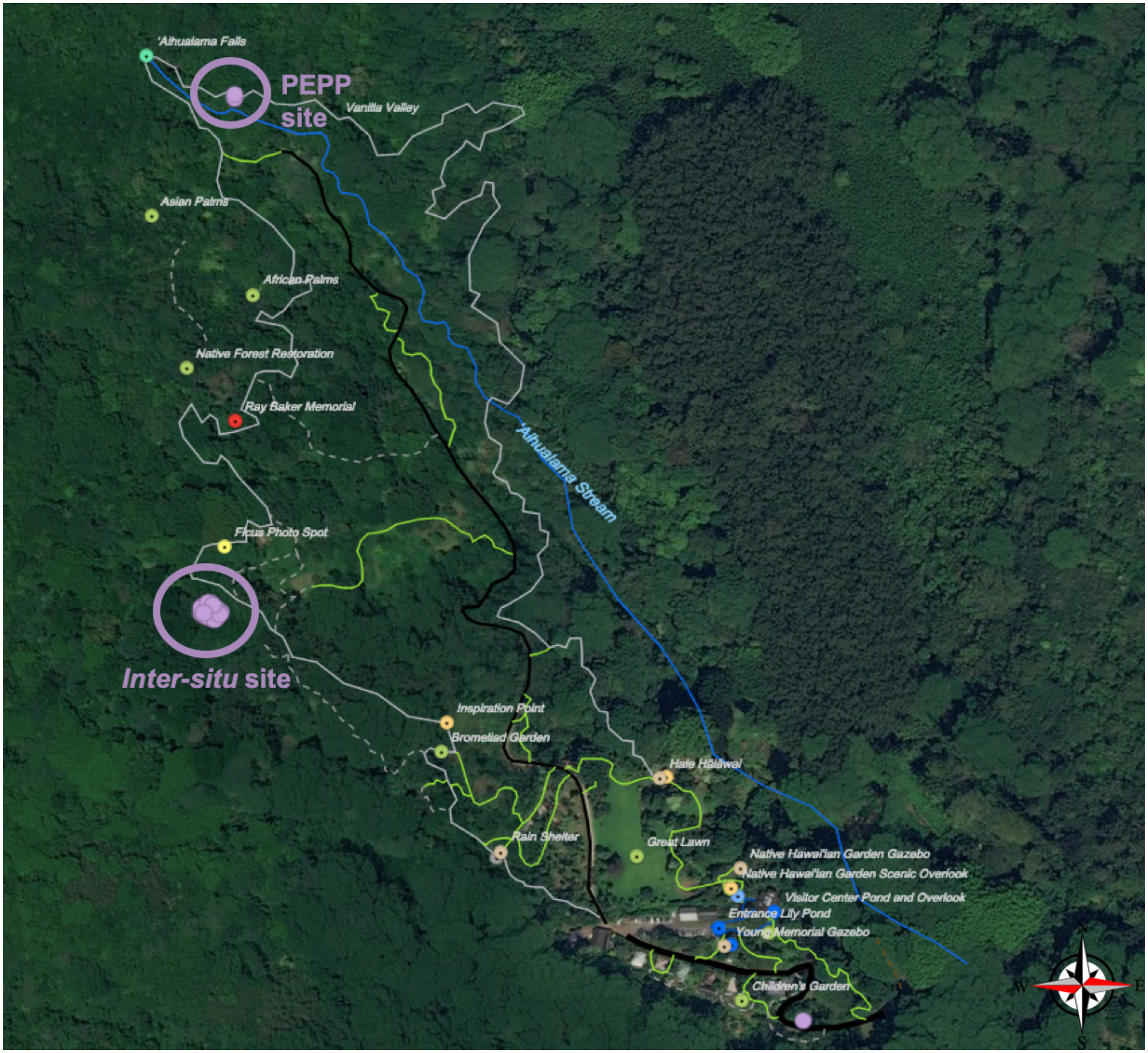

Figure 3.2: Satellite image from Google Earth of Harold L. Lyon Arboretum Grounds. Experimental site location symbolized by grouping of light purple circles. These circles are mapped polygons of each outplanted *Cyrtandra kaulantha* individual.

3.2.2 Experimental Design

Surviving *Cyrtandra kaulantha* individuals from the greenhouse experiment (n = 35), were outplanted at the ‘Aihualama site in a completely randomized design. All individuals were planted ~1m apart in a 8m × 8m grid. No fertilizers or amendments were added during outplanting, but weedy plants were cleared from the plot except for trees >2.5cm diameter at breast height. Once outplanted, there was no further management. Plants were monitored and the same data collected in the greenhouse study were collected every 4 weeks for 12 months.

3.3 STATISTICAL ANALYSIS:

Data collection of greenhouse and field study trials utilized the same methodologies and included survival rate, basal diameter, plant height, total Leaf Area and total Leaf Number of each individual plant.

Absolute growth of each variable was calculated using the equation:

$$\triangle \boldsymbol{X_t} = X_f - X_i$$

X_f = Final size by measurement
X_i = Initial size by measurement

Data were tested for normality via the Shapiro-Wilks test and visual illustration with QQ-Plots in the {stats} R package. Average absolute survival rates by treatment were non-parametric and were compared via generalized linear modeling and run through ANOVA, type III analysis (R package{stats}). Average absolute growth of each morphological measurement was compared using the Kruskal-Wallis test or general linear models with ANOVA (Type 3) analysis depending on the normality of data (R package {car} and R package {stats}). If ANOVA values were significant, pairwise comparisons were analyzed via the Tukey HSD post-hoc test. If Kruskal-Wallis test values were significant, a pair-wise Dunn Test was used to identify specific significance between treatments for unequal sample sizes (R package {dunntest}). All analyses were done in R version 3.6.1.

CHAPTER 4. RESULTS

4.1 GREENHOUSE STUDY RESULTS

4.1.1 Morphological Measurements and Survival Rate

There were no differences in the average absolute growth of *Cyrtandra kaulantha* individuals between treatments groups for any of the morphological measurements over the duration of the greenhouse experiment (leaf area (chi-squared = 3.7945, df = 4, p = 0.36), basal diameter (χ^2 = 3.2, df = 4, p = 0.52), or height (chi-squared = 4.8917, df = 4, p-value= 0.30)) (Fig. 4.1.1). Survival rates of *Cyrtandra kaulantha* plants varied by treatment over the 10-week greenhouse study (Fig.4.1.2). All treatment groups experienced mortality events 2 weeks after being repotted, except for individuals grown in the ʻAihualama treatment. This initial dieback could be due to transplant shock. However, overall differences in final average survival rates between treatments were not significant (chi-squared = 8.11, df= 4, p = 0.08). Individuals under the whole soil treatment groups had survival rates of : ʻAihualama, *S*=100% and Waikāne, *S*=80%. The Control, Phosphate and ʻAihualama + Phosphate treatment groups had survival rates of : Control, *S*=66%, Phosphate, *S*=44%, ʻAihualama + Phosphate, *S*=44%.

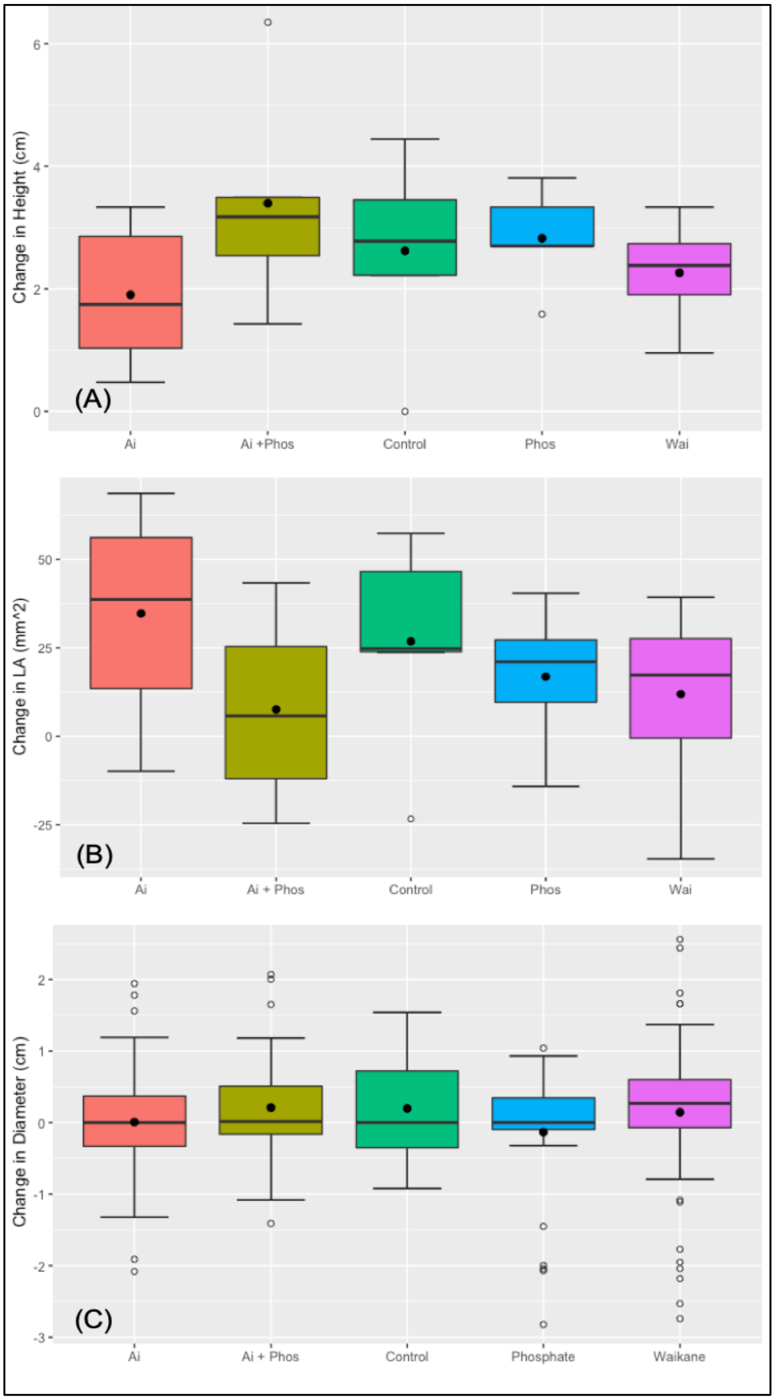

Figure 4.1.1 Boxplots of the average absolute change of morphological measurements by treatment (Ai=ʻAihualama Whole Soil, Ai+Phos= ʻAihulama Whole Soil and Phosphate, Control, Phos= Phosphate Treatment, and Wai= Waikāne Whole Soil) from the beginning to end of the Greenhouse Experimental study. (A) Change in Leaf Area (mm^2), (B) Change in Height, (C) Change in Basal Diameter. Outliers are represented by empty circles and means of each treatment are represented by black dots

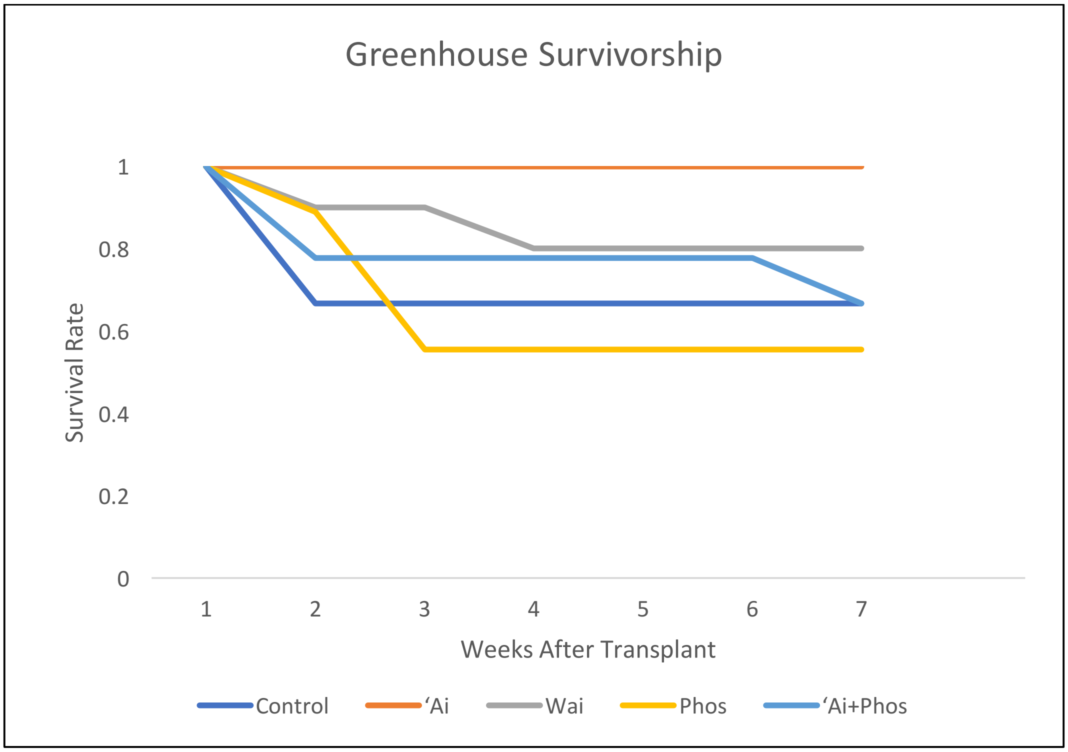

Figure 4.1.2 Percent survival of *Cyrtandra kaulantha* plants by treatment group; Control, ‘Aihualama, ‘Aihualama + Phosphate, Waikāne, and Phosphate

4.1.2 AMF Staining

Of the 10 root samples taken of each individual by treatment, only individuals in the ‘Aihualama Whole Soil Treatment showed evidence of AMF colonization (Fig. 4.1.3). These data were quantified as presence-absence only. All other treatment groups did not demonstrate colonization.

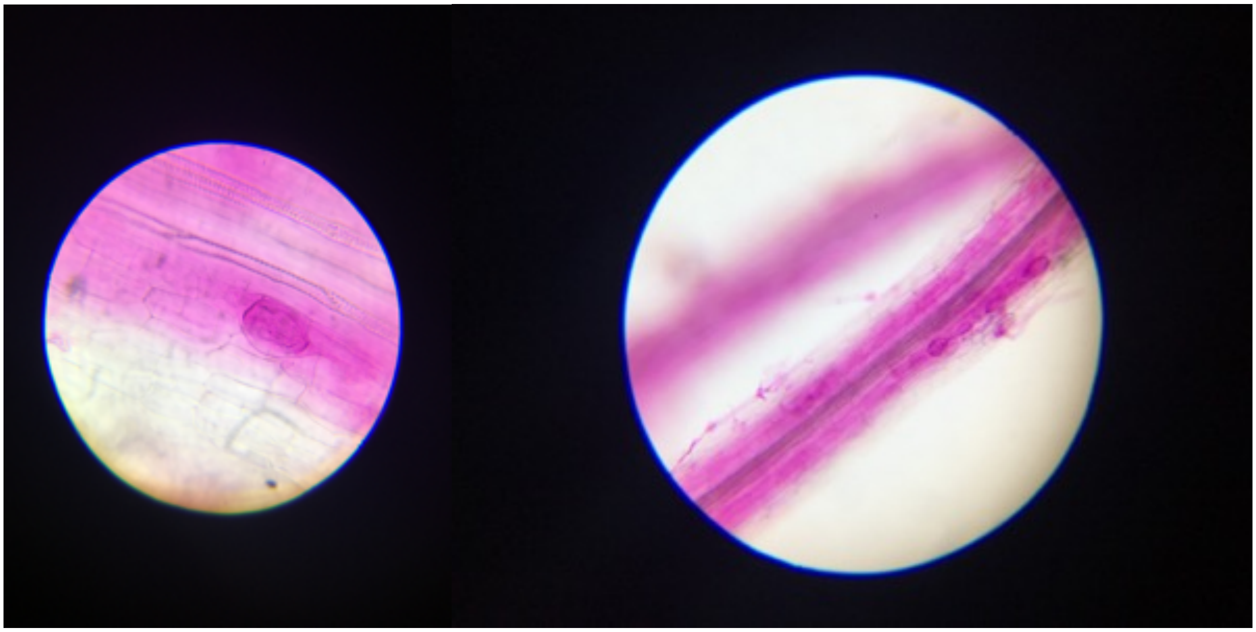

Figure 4.1.3. Example of Microscopic analysis of VAM revealing colonization of fungal mycorrhizal vesicles in the ‘Aihualama Whole soil treatment individuals

4.2 *INTER-SITU* STUDY RESULTS

4.2.1 Survival Rate

Over the 11 month duration of the field study, *Cyrtandra kaulantha* individuals in the ‘Aihulama Whole Soil Treatment (AWS) and Waikane Whole Soil Treatment (WWS) suggested low rates of mortality (AWS, *S*=90%, WWS, *S*=100%). Individuals in all other treatments suggest trends of higher mortality, with the ‘Aihualama Whole Soil + Phosphate Treatment (AP) demonstrating the lowest rate of surivorship (*S*= 50%, Fig. 4.2.1). The overall survival rate after the 12-month field growth period of all 35 individuals transplanted in the field study was 80% (regardless of treatment). This is much higher than the survival rate of the preliminary study where total survivorship was only 33% after 12-months. Similarly, all treatment groups also had higher surival rates than the individuals in the preliminary study.

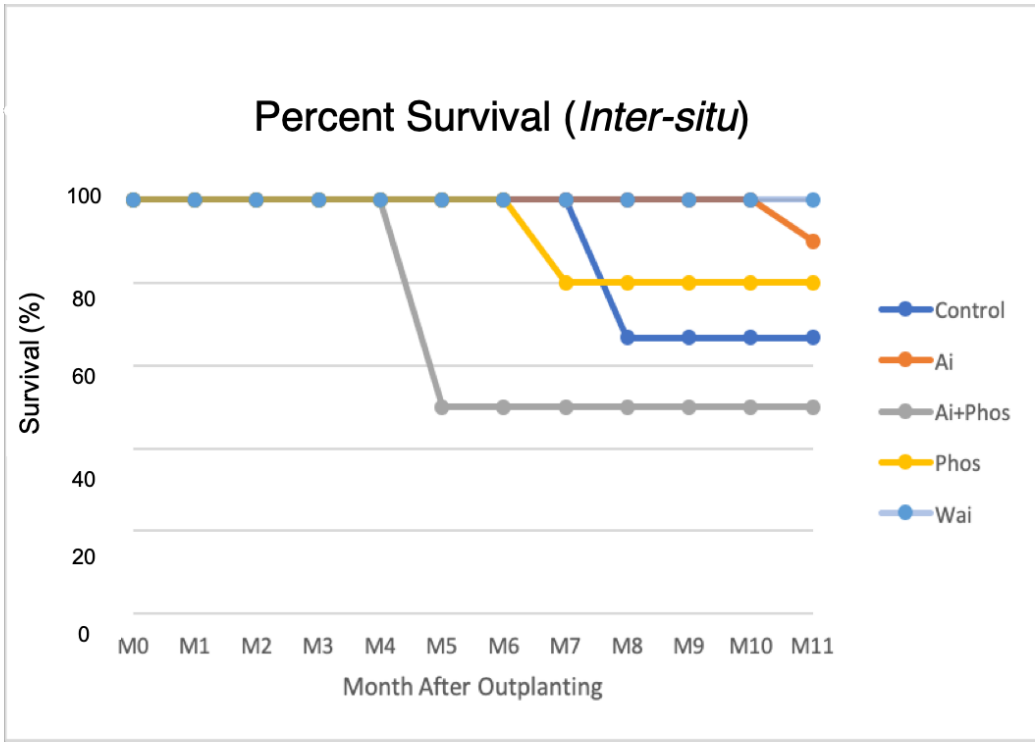

Figure 4.2.1. Survival rate over 11 month (*inter-situ)* experimental field trial of *Cyrtandra kaulantha* individuals grouped by soil amendment treatment.

There were no significant differences in the survival rates of *Cyrtandra kaulantha* individuals between treatments from beginning to end of the field study (chi-squared= 7.56, df= 4, p= 0.108). However, with such a small sample size, a p-value of 10% is suggestive of potential differences masked by conservative statistics.

4.2.2 Greenhouse to Field Survival

The survival rate of individuals by treatment from the beginning of the greenhouse study to the end of the field study, showed significant differences (chi-squared = 9.6506, df = 4, p-value = 0.047). Use of the Dunn Test for pair-wise comparisons, identified differences between the A (*S*=90%) and the AP treatment (*S*=0.33%; chi-squared = 9.65, df = 4,p -value= 0.006), the A and Control (*S*= 0.44%; chi-squared = 9.65, df = 4,p-value=0.023) and A and P Treatments (*S*=0.44%; chi-squared = 9.65, df = 4, p-value=0.023) (Fig. 4.2.3, Table 4.1). Although both the ‘Aihualama Whole Soil Treatment (*S*=90%) and Waikāne Whole Soil Treatment (*S*=80%) had the highest survival rates, W was only significantly different from individuals in the AP Treatment (chi-squared= 9.65, df=4, p-value=0.023, Table 4.1). There was no significant difference between the local ‘Aihualama Whole Soil Treatment group and the reference Waikāne Whole Soil Treatment groups. (Table 4.2.2)

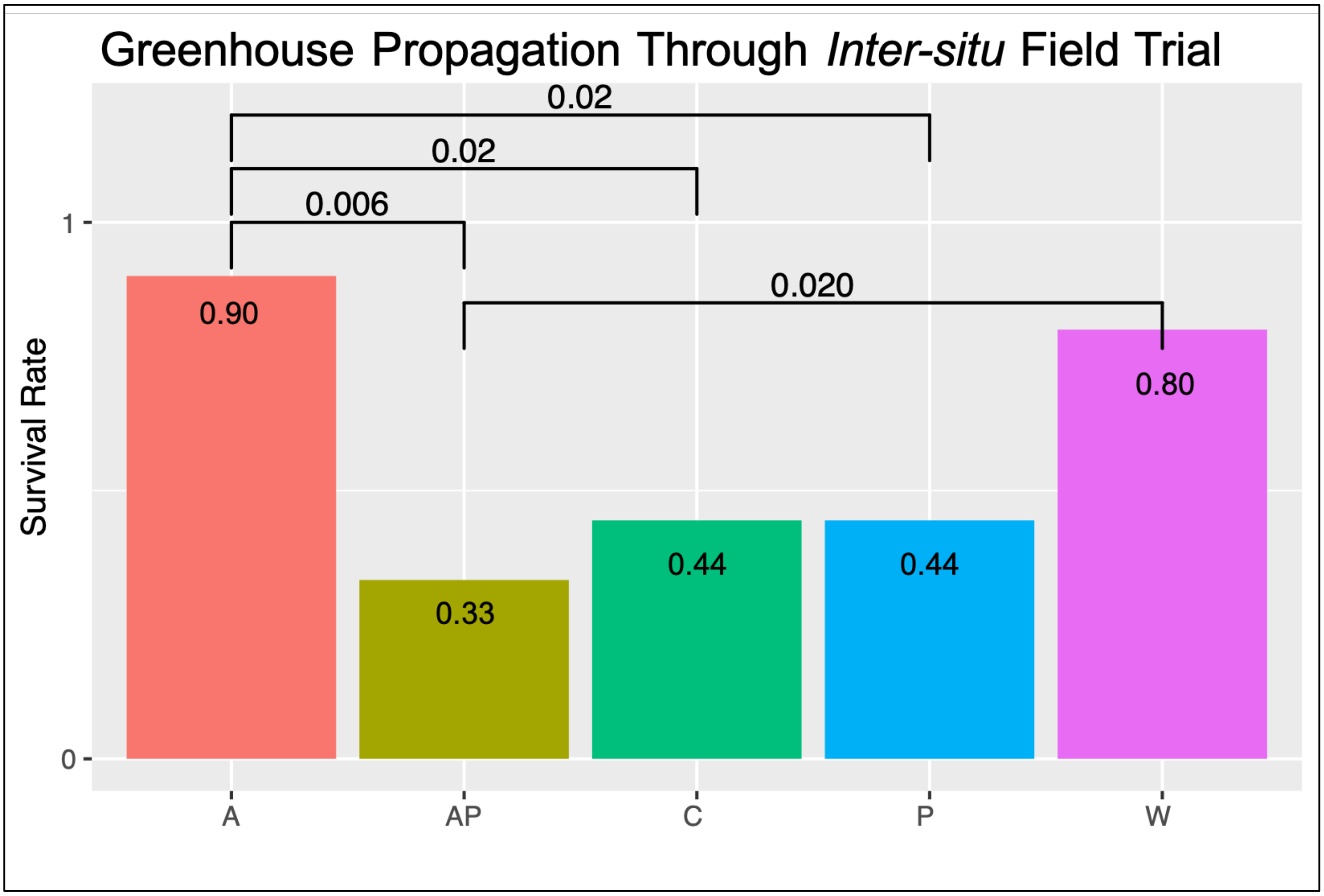

Figure 4.2.2. Average survival rate of *Cyrtandra kaulantha* individuals by treatment(A=‘Aihualama Whole Soil, AP= ‘Aihulama Whole Soil and Phosphate, C=Control, P= Phosphate Treatment, and W= Waikāne Whole Soil) over the duration of the entire experiment; from beginning of greenhouse experiment to the end of the field experiment. Overall p-value as determined by Kruskal- Wallis test is displayed above columns; brackets represent significant pairwise differences between treatments (Dunn test).

Table 4.1 Pair-wise comparisons via the post hoc Dunn Test for survival rates by treatment group (‘Aihualama Whole Soil (A), ‘Aihualama Whole Soil + Phosphate (AP), Control (C), Phosphate and Waikāne Whole Soil (W)) from the beginning of the greenhouse study to the end of the *inter-situ* study.

Treatment	A	AP	C	P
AP	**0.007***			
C	**0.023***	0.317		
P	**0.023***	0.317	0.5	
W	0.326	**0.023***	0.059	0.059

4.2.3 Morphological Measurements

As with the greenhouse study, Leaf Area (F = 0.7595, df=4, p-value= 0.5623), Stem Height (chi-squared = 3.6871, df = 4, p-value = 0.45), and Basal Diameter (df=4, F-value= 0.5945, p-value= 0.6703) demonstrated no difference in average absolute growth between treatment groups (Fig. 4.2.4). Average absolute growth for the Number of Leaves Per Plant also showed no significant difference between treatment groups (df=4, F-value =2.5723, p-value= 0.06484).

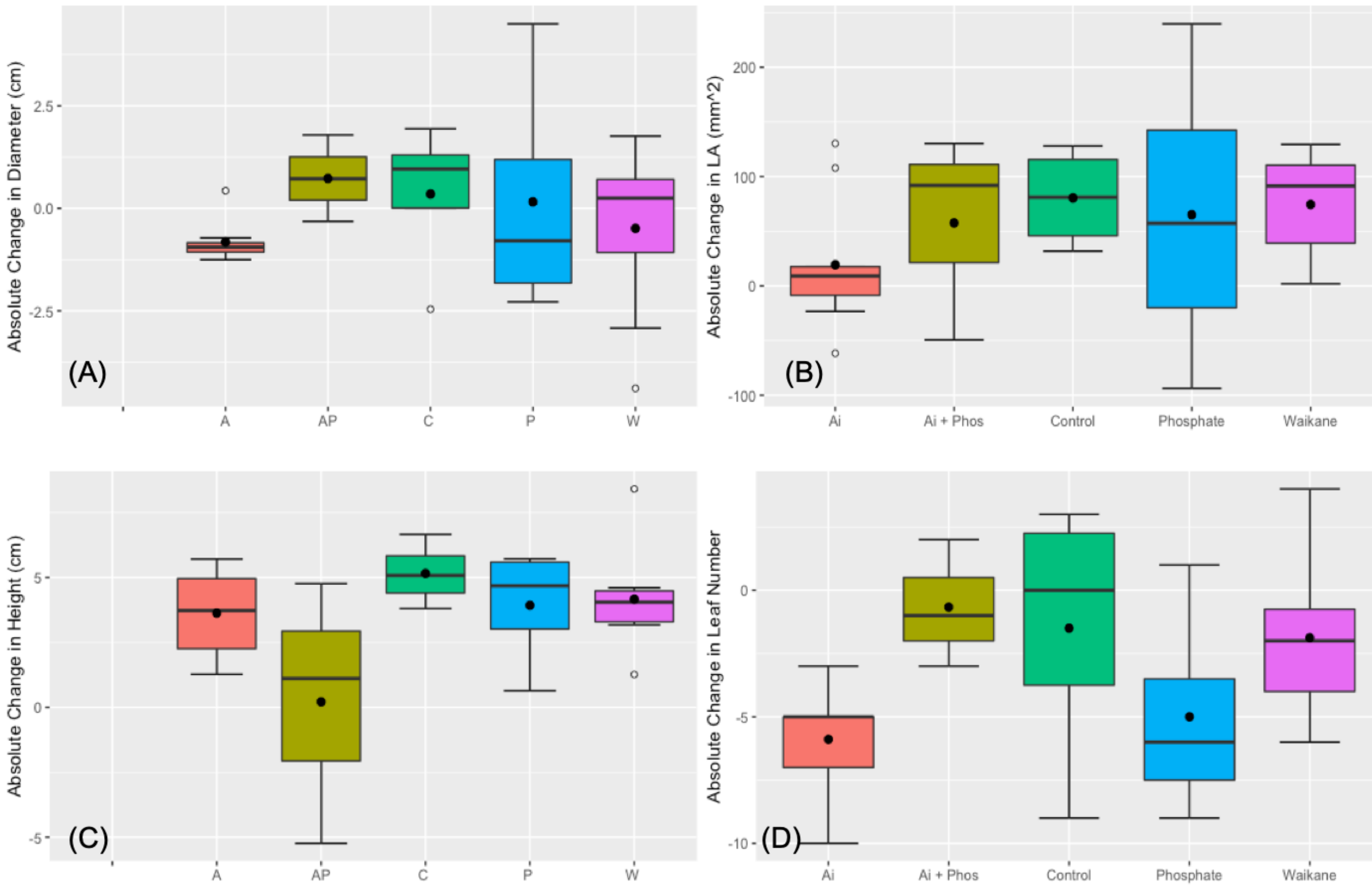

Figure 4.2.4. Boxplots of the average absolute change of morphological measurements by treatment (Ai = ʻAihualama Whole Soil, Ai+Phos = ʻAihualama Whole Soil and Phosphate, Control, Phos= Phosphate Treatment, and Wai = Waikāne Whole Soil) from the beginning to end of the *Inter-situ* Experimental study. (A) Change in Diameter (mm), (B) Change in Leaf Area (mm^2), (C) Change in Stem Height (cm), (D) Change in Leaf Number. Outliers are represented by empty circles and means of each treatment are represented by black dots.

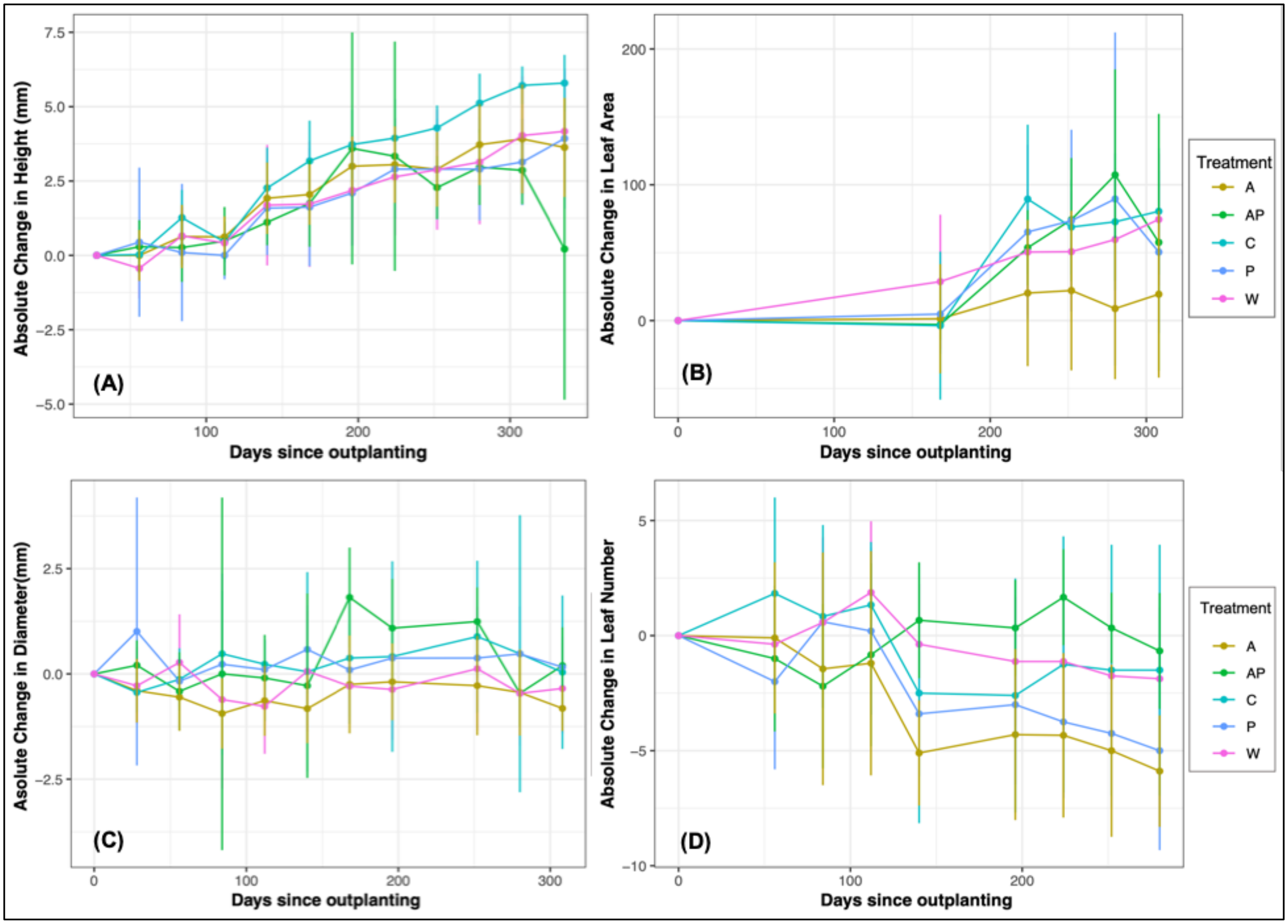

Figure 4.2.5 Graphical representation of average absolute change of morphological measurements by treatment (A=ʻAihualama Whole Soil, AP= ʻAihualama Whole Soil and Phosphate, C=Control, P= Phosphate Treatment, and W= Waikāne Whole Soil) over time from the beginning to end of the *Inter-situ* Experimental study. (A) Change in Stem Height (cm), (B) Change in Leaf Area (mm^2), (C) Change in Basal Diameter (mm), (D) Change in Leaf Number. Standard deviation of the mean for each date was calculated, represented by vertical bars.

Relative Growth Rate by Height (RGR_H) was calculated using the standard equation:

$$RGR_H = \frac{\ln(Height_2) - \ln(Height_1)}{(t_2 - t_1)}$$

All treatment groups had the largest fluctuations in average Relative Growth Rate by Height (RGR_H) over the first 4 months after outplanting. By the 5th month, average RGR_H became fairly constant at about 0.015 $mm.m^{-1}.day^{-1}$ across all treatments (Fig. 4.2.5). Average RGR_H of individuals in the ‘Aihualama Whole Soil + Phosphate Treatment declined in the last month of data collection, giving the only negative relative growth rate at the end of the *inter-situ* study. The average RGR_H from the beginning to the end of the *inter-situ* study showed no significant differences between treatment groups (chi-squared = 4.7493, df = 4, p-value = 0.314).

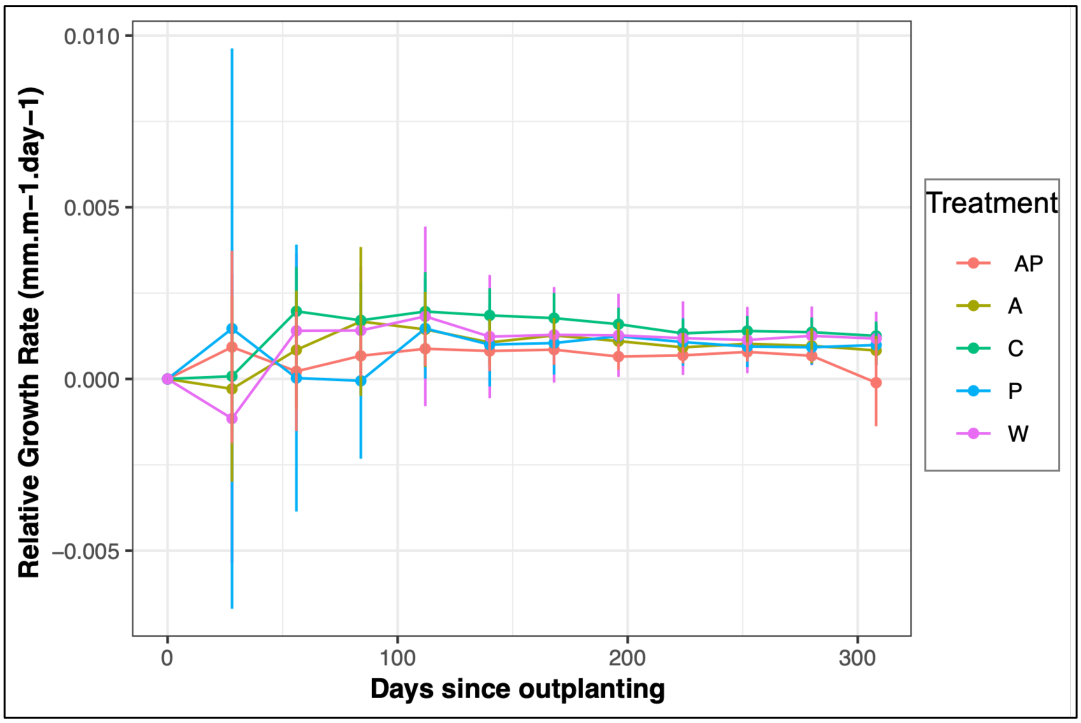

Figure 4.2.5 Relative Growth Rate of *Cyrtandra kaulantha* based on stem height by treatment group (A=‘Aihualama Whole Soil, AP= ‘Aihualama Whole Soil and Phosphate, C=Control, P= Phosphate Treatment, and W= Waikāne Whole Soil) over the duration of the *inter-situ* field study

At the end of the field study, *Cyrtandra kaulantha* individuals across treatment groups had similar average morphological characteristics. Average leaf number per plant by treatment ranged from 6-11, average leaf area ranged from 93.50mm^2 to 174.80mm^2, average heights ranged from 13.65mm to 16.51 mm and average stem diameter ranged from 10.69mm to 11.99mm (Table 4.4). None of the plants flowered or fruited, thus, all are likely still juvenile.

Table 4.3. Comparison of average final growth of *Cyrtandra kaulantha* per variable by treatment at the end of the field study.

Treatment	Final Leaf Area (mm^2)	Total Number of Leaves	Final Height (cm)	Final Diameter (mm)
‘Aihualama WSI (n=9)	93.50	6	15.97	11.06
Waikāne WSI (n=8)	127.37	9	14.49	11.61
‘Aihualama WSI + Phos (n=3)	174.80	8	13.65	11.99
Phosphate (n=4)	124.31	8	15.28	10.69
Control (n=4)	165.34	11	16.51	11.37

CHAPTER 5. DISCUSSION

5.1 GREENHOUSE IMPLICATIONS

One of the main goals of this research was to track the survivorship and growth of *Cyrtandra kaulantha* from the beginning of the propagation stage to the end of the field study. Plants typically invest the first several weeks of growth into root development after transplanting in response to root damage and stress. Therefore, if is unsurpising that the 10 week greenhouse study showed limited differences across any of the morphological measurements between treatment groups. The greenhouse study was largely a means to set up the field study by allowing interactions between whole soil microbiota and developing roots. Root colonization is most effective in young, newly forming roots, thus making the propagation and cultivation stage critical to the methodology of the *inter-situ* field study and the study at large. Many studies focus either on greenhouse or field methodologies but for endangered species the transition from *ex-situ* cultivation is a critical step.

Introducing inocula in the greenhouse may not have observable impacts on the bench, but can greatly impact succesful transplantation. If effective, inoculation during the greenhouse phase is considerably more economical than inoculation in the field. *Ex situ* inoculation requires smaller quantities of soil, which decreases distrubance to collection sites, and decreases the amount of physical labor needed to transport soil to remote locations. The introduction of whole soil inocula during this phase is low cost, and low maintenance.

5.2 AMF ROOT COLONIZATION

Individuals grown in the ‘Aihualama Whole Soil treatment demonstrated trends of highest survivorship (in both the greenhouse and overall study) and were also the only individuals by treatment with confirmed presence of AMF colonization after the greenhouse trial. This finding may suggest that the presence of a mutualistic relationships between *Cyrtandra kaulantha* and certain local fungi increases the chance of survivorship from the propagation stage to the 1st year of outplanting. Relationships with fungal symbionts can decrease the negative impact of transplant shock, dehydration, and herbivory that occur in both the *ex-situ* and *inter-situ* environment (Idol and Diarra 2017). Interestingly, individuals in the ‘Aihualama Whole Soil + Phosphate Treatment did not show presence of AMF colonization, which was expected due to the inhibitory effects of phopshate fertilizer at about 150ppm (Osorio and Habte 2001, Dumroese 2012). It is pertinent to note, however, that the absence of AMF colonization in the root samples of other individuals does not definitively mean that they did not form relationships with AMF, just that they were not present in surveyed subset. Additionally, the success of individuals in the ‘Aihualama Whole Soil treatment group may have also been enhanced by unquantified abiotic or biotic interactions; including benefits from unspecificied plant growth promoting rhizobacteria or other microorganisms.

Whether or not individuals in the ‘Aihualama Whole Soil Treatment demonstrated higher survivorship due to AMF, the identified presence of colonization has direct implications for the future restoration of *Cyrtandra kaulantha*. Our root staining experiment revealed the first ever documentation of root colonization in this species; 90% of native hawaiian plants are believed to be dependent on AMF and thus co-evolution of *Cyrtandra* sp. and fungi should not be suprising (Gemma, Koske and Flynn 1992). Now that this relationship has been formally detected, *Cyrtandra kaulantha* could be a candidate for futher research into the benefits of AMF inoculation for improved growth and survival.

5.3 SURVIVORSHIP

The survival of reintroduced species is a critical paratmeter of success in restoration. Initial survival is an important, though often unquantified, milestone in effective establishment of sensitive species. This milestone can be inhibited by the effects of transplant shock or dessication in the first couple of months (Salama et al. 2018). Once established, long term survival, especially of individuals that have reached maturity, becomes critical for population resiliency and natural regeneration (Maschinski & Quintana-Ascension 2016). All outplanted *Cyrtandra kaulantha* individuals survived until the first mortality in month 5, therefore likely avoiding deleterious effects of transplant shock.

The longer-term survival rate of *Cyrtandra kaulantha* individuals for the *inter-situ* study were not significantly different for different treatments. However, with such small samples sizes (i.e. ‘Aihualama + Phosphate, n=3), a p-value of 0.1 estimated during a preliminary study is suggestive of possible differences in survival rate between treatments. These results justify further research. Differences in the survivorship rates of *Cyrtandra kaulantha* by treatment approached significance from the propagation stage through the end of the field study. *Cyrtandra kaulantha* individuals in the ‘Aihualama Whole Soil and Waikāne Whole Soil Treatment group demonstrated a trend toward higher survival rates, which if true would support our original hypothesis that individuals grown in either ‘Aihualama Whole Soil treatment group or the Waikāne Whole Soil treatment group would have higher rates of survival. This would need to be determined by future research.

Both whole soil treatments were inclusive of the soil microbiota that naturally occured at either site. As described in the literature review, the presence of plant growth promoting rhizobacteria (PGPR) and fungal symbionts like arbuscular mycorrhizal fungi (AMF) in whole soil inocula can potentially aid in plant growth (Velivelli et al. 2015). Numerous strains of PGPRs can increase resiliency to diseases by enhancing a plant's systemic resistance as well as providing external protections via the parasitizing of deleterious nematodes and fungi (Kloeppe et al. 1999; Magdorf and Van Hes 2009). Studies that have evaluated the use of whole soil inoculants have found significant correlations between improved plant vigor and addition of PGPRs by inoculation (Ji et al 2010). AMF is a well documented plant growth enhancer and can reduce the negative impacts of abiotic stressors, such as fluxuations in water availability, which occured regularly over the duration of this study. Furthermore, there may be positive interplay between PGPRs and AMF because rhizobacteria can benefit from increased surface area as a result of AMF root colonization (Velivelli et al. 2015). *Cyrtandra kaulantha* individuals that received whole soil inoculation may have benefited due to the their enhancement by microbiota found at both the ʻAihulama reference site and the Waikāne reference site.

Although the ʻAihulama + Phosphate Mixed Effects Treatment had the same amount of whole soil as the ʻAihualama Treatment amendment, survival rates of individuals in this group were lower than the other whole soil treatments, although the low sample sizes make definite conclusions difficult. Lower survival rates could be due to inhibition of AMF root colonization during the critical growth phase that otherwise would have enhanced plant resiliency. Phosphorous levels of 150ppm or higher have the ability to suppress mycohrrizal development (Dumerose 2012; Lonegran and Cripps 2013) and therefore could have negated the potential positive effects that the ʻAihualama Whole Soil inoculum may have provided.. Although individuals may eventually form relationships when new roots begin to develop in the field, the process is slowed and can leave plants vulnerable at a critical establishment phase. Individuals in the mixed effects treatment and Phosphate Amendment treatment had low survival rates, suggesting that the amendment of phosphrous rich fertilizers in the green house does not necessarily enhance survival rates of *Cyrtandra kaulantha* once outplanted.

Lack of differences between the Waikāne reference whole soil inoculum and the ʻAihualama reference whole soil inoculum leave questions about the differences in composition of the microbial community at each site. Degradation of habitat often creates lasting legacy effects that alter the soil condition and change microbial community structure (DʻAntonio, August-Schmidt, and Fernandez-Going 2016). Our study did not quanitfy these differences, but additional information about the interaction between *Cyrtandra kaulantha* and whole soil inocula could be elucidated by running genetic analysis on soils from both sites. It is possible that the composition of microbiota in the soils were similar or contained the same critical symbionts. Conversely, *Cyrtandra kaulantha* may not be as discerning or specific in terms of mutualistic associations and may not require microbial interactions with species from the same micro-habitat that it evolved in. Hawaiian *Cyrtandra* species are phenologically similar, if not identical, and indigenous across all islands (Johnson et al. 2019) and accordingly, may have common symbionts. It is possible that the whole soil inocula contained an unidentified abiotic or biotic property that positively impacted the survival rates of individuals amended under the treatment. If soil microbial communities collected from adjacent habitats (e.g., the ʻAihualama study site) can be as beneficial to native plant enhancement as microbiota from the reference site, this further promotes the use of soil inoculation as an appropriate methodology. Extracting soil from a reference habitat can be viewed as destructive and therefore is limited in application (Koziol et al 2017). If there are no significant differences between reference and *inter-situ* sites, whole soil inoculum can be created from local soils, leaving reference habitats undisturbed.

Although increased survivorship rates of *Cyrtandra kaulantha* approached significance with whole soil inoculum, it is pertinent to note that any differences caused by the whole soil inoculum additions could be from any number of unidentified soil properties or interactions. It will be important to parse out the specific mechanisms through more specialized studies. Additionally, the average age of reproductive maturity for the closely related Hawaian species, *Cyrtandra dentata*, is 6 years (Bialic-Murphy, et al 2017). Assuming a similarity between the species, *Cytandra kaulantha* presumably needs at least 5 years to flower once outplanted. To truly identify the success of our outplanting site, survivorship data should continue to be collected over the next 4-5 years.

5.4 MORPHOLOGICAL MEASUREMENT INTERPRETATION

Our study did not support the hypothesis that *Cyrtandra kaulantha* individuals in the ‘Aihualama or Waikāne Whole soil treatment groups would have signifcantly larger increases in morphological growth over the field study period when compared to other treatments. However, it is interesting to note that individuals amended with Phosphate also showed no significant increase in morphological growth over the study period. Phosphorous rich fertilizers are almost always added to plants in the cultivation phase to enhance growth. These amendments are commonly considered beneficial to native Hawaiian plants by providing enhancement to plant fitness and development (Dumerose, Davis and Jacobs 2011).This conventional method is often used in the greenhouse as a means of increasing *in situ* survivorship (Salifu and Timmer 2003).

Contrary to this widely accepted methodology, *Cyrtandra kaulantha* individuals did not have a significant or lasting growth response to the addition of rock phosphate. This finding suggests it is possible that *Cyrtandra kaulantha* is not as limited by phosphorous as other species, potentially due to evolutionary adaptations caused by the low plant available phosphorous content in highly weathered Hawaiian soils. This facet is important to explore further because it could impact *ex situ* greenhouse cultivation practices and have financial implications for organizations that spend money on phosphorous rich fertilizers. Additionaly, the amendment of phosphate in the greenhouse may benefit plants in cultivation, but lead to disadvantageous effects in the field. When phosphorous is readily available to young plants, mutualisms are less likely to form, leaving these same plants more vulnerable when they are outplanted into harsh enviroments with less accessible nutrients (Lonegran and Cripps 2013).

Morphological measurements (LA, basal diameter, leaf number, height) demonstrated no treatment differences for the greenhouse, field, or total study. Working with rare and endangered species always poses challenges due to inherent limitations in access to plant material (Bialic-Murphy 2017). Because of the permitting and restrictions placed on federally listed species, a small sample size is almost certainly a factor that limits accurate assessments of growth and development as it is likely that many treatment effects across the study would be undetectable at such a low sample size. It is possible that morphological growth may have been different between treatments, but was undetectable at such low replication. Additionally, *Cyrtandra kaulantha* individuals were impacted by herbivory at the field site, which may have impacted the overall growth of the plant as well as the accuracy of measurements (such as leaf area). Accounting for the effect of herbivory could be important to understanding the aboveground morphology of the species and which measurements can be accurately used to quantify growth.

5.5 SITE CONSIDERATIONS

When the field study plot site was intially selected, several factors were measured to quantify similarities between the reference site (Waikāne Valley) and the *inter-situ* outplanting site including the density of canopy cover. Intial readings showed both sites to have similar canopy cover values, however, the upper right quadrant of the *inter-situ* site is located at the base of three large *Toona cilata* trees, with several large *Ficus* upslope. Of the in-field mortality, 5 of the 7 *Cyrtandra kaulantha* individuals that died were outplanted in the upper right quadrant of the plot. Although we tried to account for heterogeneity in our site via randomization, it is possible that seasonal differences and their subsequent effects may have impacted the outcome of this study.

Throughout the year, overstory trees undergo different stages in their life cycle. *Toona ciliata,* a tree located above the upper right quadrant of the *inter-situ* site, is a decidudous tropical species and may have developed a dense canopy that decreased light penetration to the understory (Taylor and Harden 1991). Light can be a strong limiting factor to understory species in shaded forest (Machado et al. 2003) Promixity to large trees may also increase interactions with seasonal leaf litter that can alter soil pH, moisture, and arthropod habitat (*Cyrtandra kaulantha* individuals planted in the upper right quadrant of this plot were more likely to encounter competition for water absorption from rhizosphere roots of large trees which could decrease fitness and resiliency in times of drought). Based on these observations, we recommend considering seasonal overstory changes when selecting a translocation site for *Cyrtandra kaulantha* in a novel habitat. *Cyrtandra kaulantha* individuals growing in the Waikāne Valley reference site, tend to grow in medium shade and therefore a site with the potential for heavier seasonal shading should be avoided. The impacts of leaf litter and nutrient competition in the rhizosphere should also be explored further.

5.6 PRELIMINARY STUDY AND INTER-SITU SITE COMPARISON

The preliminary study site created by PEPP and HRPP at Harold L. Lyon Arboretum, was not monitored or quantified in as rigorous a manner as our *inter-situ* study site. However, general comparisons are possible in attempts to identify parameters for site selection when restoring *Cyrtandra kaulantha*. The overall survivorship of *Cyr kau* at our *inter-situ* site (S=80%) was much higher than at the perliminary site (S=33%). This could be due to the substrate and location of the selected restoration site. Like many Hawaiian *Cyrtandra* species endemic to the Koʻolau Mountains (Bialic-Murphy 2017), *Cyrtandra kaulantha* is known to grow primarily on rock walls and in the bottoms of gulches. The preliminary study site selected by the PEPP and Lyon Arboretum managers was adjacent to the ʻAihualama stream but the substrate was predominantly of clay soils on the embankment. When selecting our *inter-situ* study site we chose to plant *Cyrtandra kaulantha* individuals in a rocky area about 3m from the edge of an adjacent ephemeral gulch. The preliminary study site also had much higher light levels which led to encroachment by basket grass (*Oplesmenus* sp.) and other successional invaders. Competition between invasive plants and *Cyrtandra kaulantha* may have decreased survival rates at the preliminary study site. Additionally, *Cyr kau* individuals at the preliminary site had an infestation of Twig Borer (*Xylosandrus compactus*) while our main study site did not. Both sites showed evidence of herbivory by slugs and other insects, however neither site demonstrated any evidence of damage by ungulates.

5.7 *INTER-SITU* RESTORATION APPLICATION

With increased reliance on reintroduction to conserve endangered flora, species specific data need to be gathered and empirically analyzed. *Inter-situ* restoration provides an opportunity to identify the parameters that most impact species success in order to create best practices. Despite the call for these data, most *inter-situ* restoration projects remain documented solely in “grey papers” because their global application is perceived as limited (Vitt et al. 2016). Although limited by small range, detailed case studies such as these can be used to examine complexities that are often overlooked in larger studies (Kruschelnycky et al. 2016). Our project is meant to highlight the uses and benefits of *inter-situ* restoration and collaboration with conservation organizations like arboreta. As more of these types of projects are shared amongst researchers and plant restoration managers, broader applications can be drawn and related to endangered species rehabilitation. The definitions of success for reintroductions are widely disparate and can be deemed at vastly different stages; from initial establishment through 2nd generation recruitment (Guerrant 2013). Thus it is important to have continous monitoring throughout the lifestages of the focal species and constant adaptation of the *inter-situ* site.

CHAPTER 6: CONCLUSIONS AND RECCOMMENDATIONS

Our study had two key findings: 1.) Our results did not support the prediction that whole soil inocula would have a significant effect on the absolute growth of *Cyrtandra kaulantha* by the end of the field study. However, our findings suggest that whole soil inocula treatments may have contributed to increased survival rates of *Cyrtandra kaulantha* from the start of the greenhouse study to the end of the field study; 2.) *Inter-situ* restoration plots can be a valid method for testing targeted quantitative questions about rare and endangered species in a semi-novel ecosystem.

6.1 Conclusion and Recommendation One: *Cyrtandra kaulantha* and AMF

Our results confirmed that *Cyrtandra kaulantha* exposed to whole soil inoculum from ‘Aihualama valley was colonized by arbuscular mycorrhizal fungi. This relationship suggests that *Cyrtandra kaulantha* may be dependent on mycorrhizal symbioses in some way for growth. Additionally, the ‘Aihualama Treatment group had a trend toward highest survival, which may have been impacted by AMF colonization. Therefore, *Cyrantdra kaulantha* could be a candidate for further research into the benefits of AMF inoculation and could potentially have increased outplanting success via improved growth and survival due to AMF relationships.

Further studies of the benefits of AMF inoculation is suggested for *Cyrtandra kaulantha* as well as other endangered Hawaiian plant species. In order to determine these relationships, DNA extraction and further root staining should be conducted by a microbiologist in order to quanifty the amount and impact of colonization by species. Our findings emphasize the need for further research into the dynamics between mutualistic soil microbes, like AMF, and endangered species like *Cyrtandra kaulantha*.

6.2 Conclusion and Reccomendation Two: Use of whole soil inocula for restoration plantings

The use of whole soil inoculum might enhance the survivorship of critical species through exposure to beneficial microbiota. Individuals grown in treatments with whole soils from both the reference site in Waikāne and the *inter-situ* site in ‘Aihualama demonstrated trends of higher survivorship than individuals in all other treatments from the propagation stage through the field study stage. We also found no differences in survival rates between individuals in the phosphate treatments. These findings suggest that whole soil inoculum could be more beneficial to the restoration success of *Cyrtandra kaulantha* than other traditional phosphorous fertilizer regimes. There were also no differences in survivorship between the ‘Aihualama and Waikāne whole soil treatment groups, suggesting similarities between the soils or lack of host specificity by *Cyrtandra kaulantha*. If supported by further studies, this finding has could have beneficial implications for whole soil collection, promoting the use of local soil instead of reference soil to decrease physical disturbance to sensitive habitats.

Future studies are needed to determine the exact causes of our observed trends. DNA analysis and characterization of fungal and bacterial communities are necessary to determine if soil microbiota is the reason for potential increase in survival rate of outplanted *Cyrtandra kaulantha*. Our research does, however, suggest trends towards increased survival in individuals grown under both whole soil inocula treatments, therefore we recommend that soil inoculation be considered in restoration outplanting projects until otherwise explained. We recommend that since there is no clear difference between effects of local and reference soil inocula, that local soil be used for inoculation because it is easier to access and less damaging to sensitive habitats.

6.3 Conclusion and Recommendation Three: *Inter-situ* site selection

Our *inter-situ* restoration site was located outside of the historical range of our study species but had similar observable biotic and abiotic conditions. Its location on the grounds of a state-owned research unit, Harold L. Lyon Arboretum, made the site easily accessible for regular monitoring and ensured the integrity of the site. Overall, *Cyrtandra kaulantha* individuals planted at our *inter-situ* site had higher survivorship rates, lower evidence of desiccation and no evidence of infestation by Twig borer, as compared with individuals grown at the preliminary study site. Additionally, *Cyrtandra kaulantha* individuals in our study were propagated in a greenhouse adjacent to the study site as opposed to the Pāhole Rare Plants Greenhouse that is located at much higher elevation. This "hardening-off" period provided by growing plants for the study near the site of planting may have reduced initial mortality due to stressors such as transplant shock when outplanted at our *inter-situ* site.

Based on these conditions, we recommend that *Cyrtandra kaulantha* and other rare plants, be given time to acclimate in an adjacent greenhouse before being reintroduced. We also recommend the use of *inter-situ* restoration as an appropriate step to identify the specific parameters that impact the success of restoration via research and manipulation without disturbing sensitive habitat of extant populations. Future studies are needed to compare the impacts of these parameters on *Cyrtandra* species between multiple *inter-situ* sites in order to draw about interacting abiotic and biotic environmental factors.

6.4 Summary

As global climate change increases extinction pressures on endangered species, novel and quanitfiable restoration practices need to be tested and identified. *Inter-situ* restoration can be utilized as a framework to help managers quickly identify best practices and create adaptive strategies after rigorous field testing. Rare plant species, like *Cyrtandra* kaulantha, may rely on the unseen biotic relationships that they form belowground to uptake nutrients and withstand shifting environmental conditions. The use of whole soil inoculation is a low cost method that could be helpful in enhancing the survivorship and growth of endemic species that depend on mutualisms to survive. Further inquiry into the specifics of these practices is necessary to increase successes from propagation to field. If the intricacies of interactions between soil microbiota and endemic species can be better understood, there may be increasingly successful reintroductions of species to areas outside of their historical range.

APPENDIX A. SUPPLEMENTARY MATERIALS

A.1. PRELIMINARY STUDY

The preliminary study at ‘Aihualama stream included 40 plantings of *Cyrtandra kaulantha* as well as plantings of two species of endemic and endangered Hawaiian lobeliads: *Cyanea truncata* and *Cyanea crispa* (Fig. A.1). Since the creation of this site, all initial plantings have died, but other common natives, such as *Pipturus albidus*, remain at the site. The site is now under the direct management of the Lyon Arboretum staff and is slowly being restored to a semi-native habitat. *Cibotium glaucum*, *Hibiscus arnottianus*, and several *Microlepia strignosa* plants have been added to the site to increase ground cover.

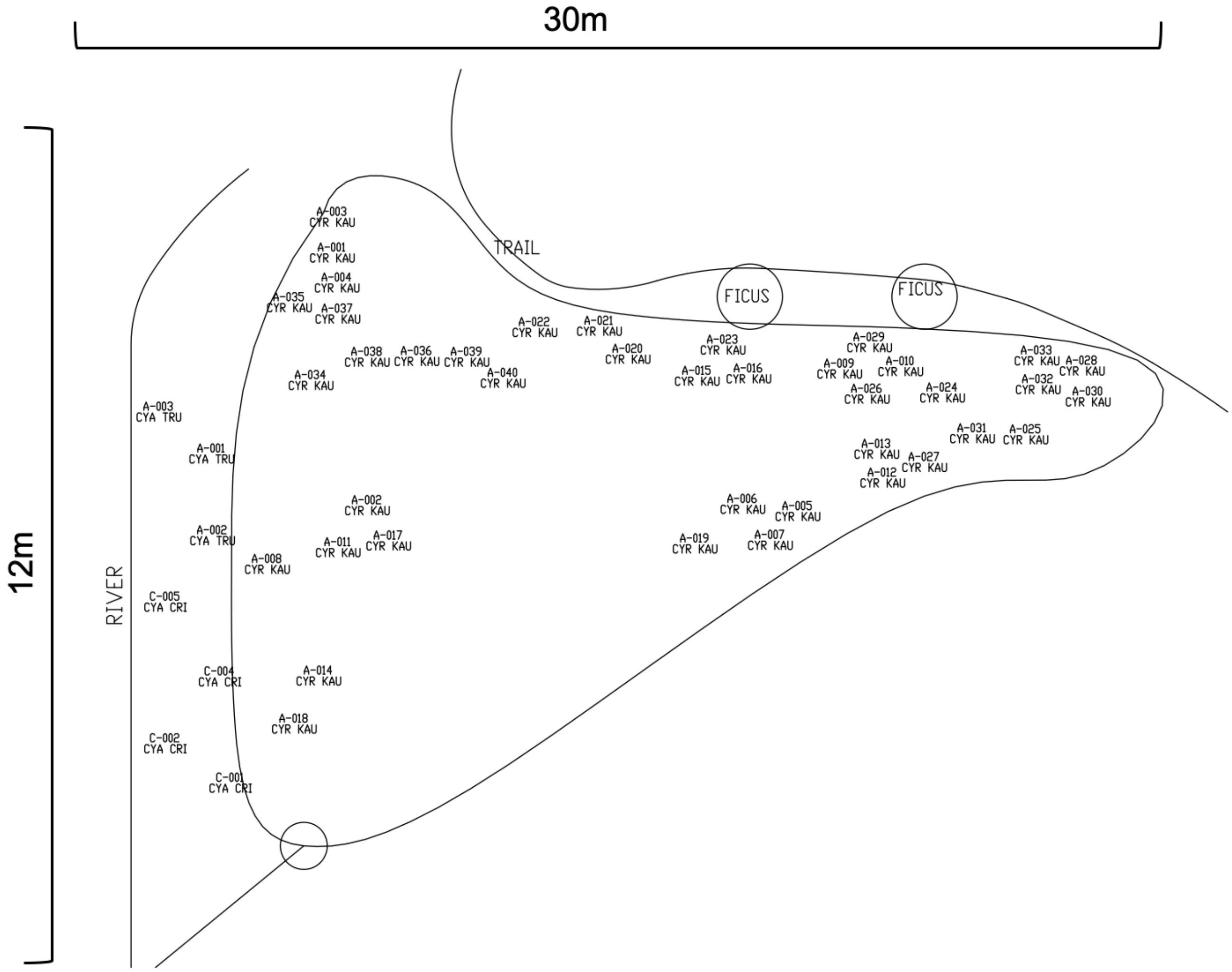

Figure A.1 AutoCad rendered map of the layout of the preliminary PEPP restoration site and locations of *Cyrtandra kaulantha* (CYR KAU) individuals as established by HRPP and PEPP in October 2016. *Cyanea crispa* (CYA CRI) and *Cyanea truncata* (CYA KAU) were also planted above the embankment.

Diagnoses of freshly predated *Cyrtandra kaulantha* individuals were brought to the Agricultural Diagnostic Service Center. Two different types of twig borer insects were identified in the chamber of the stem. Due to infrequent monitoring at the preliminary study site, it is unknown if the infestation was the cause of plant mortality or a symptom of a pre-existing complication. Twig borer insects often are attracted to plants that are already beginning to decompose and are common pests of hardwood trees like *Theobroma cacao*. This is the first documented occurrence of twig borer in *Cyrtandra kaulantha*, a rare find in semi-woody, herbaceous shrubs.

Table A.1 Insect Diagnosis Results of Twig Borer pest infestation in *Cyrtandra kaulantha* collected from the preliminary *inter-situ* restoration site

University of Hawaii at Manoa
College of Tropical Agriculture and Human Resources
Agricultural Diagnostic Service Center
INSECT DIAGNOSIS RESULTS
26-Sep-17

CLIENT:
Lyon Arboretum
3860 Manoa Road
Honolulu, HI 96822

atten: Pia Ruisi-Besares

JC# 2018-054803
Sample No: 2018-054803-002
Lab No: 17-050

E-Mail: pruisibe@hawaii.edu

PHONE: Business: (808) 988-0466 Home: (808) **Cell:**

Recv'd by ADSC: 13-Sep-17 Recv'd by Clinic: 13-Sep-17 Completion: 26-Sep-17

Coll.Site: Manoa Valley **Island** Oahu **Coll Date:** 9/13/17

HOST: **Code**: FO **Name:** Cyrtandra kauaiensis ?

Sample Description/ Symptoms:

Terminal branch approx. 19" with terminal leaves. Diameter of branch 3/8" to 1/2". Tiny borer holes along length of branch. Cut off approx. 5" off bottom branch to open up and search for beetles.

Pest Identification:

Recovered 7 small beetles and several eggs and grubs.

1) Xylosandrus compactus - black twig borer	COLEOPTERA: Ptinidae: Scolytinae	5 adults
2) Xylosandrus morigerus - a twig borer	COLEOPTERA: Ptinidae: Scolytinae	2 adults

Results/Recommendation

Not too much information on #2 X. morigerus

Black twig borer - important pests on numerous ornamental and food crops.

A.2 RELATIONSHIPS BETWEEN SIZE, GROWTH, AND SURVIVAL

Studies have suggested that survival rates after transplant can be impacted by the size of the individual at time of planting. We found no differences of the effect of initial height by treatment for any of the morphological measurements. This is likely due to the fact that plants were vegetatively propagated and thus intentionally did not vary much in size when planted (Table A.2).

Table A.2 Effect of inital size at outplanting by treatment on survival rate of *Cyrtandra kaulantha* individuals from the beginning to the end of the *inter-situ* field study.

Measurement	R (^2)	F Stat	DF	P-value
Initial Height by Treatment	0.2086	1.476	5, 28	0.2292
Initial Diameter by Treatment	0.0656	1.464	5, 28	0.2331
Initial Leaf Area by Treatment	0.2173	1.61	5, 29	0.1887
Initial Leaf Number by Treatment	0.1989	1.44	5, 29	0.2398

The relationship between initial height at the time of outplanting and the absolute change in height were compared with linear regression analysis. The results suggest that there is no significant relationship between the two(R^2=0.002, p-value=0.83, Fig. A.2), suggesting that height at the time of outplanting was not a confounding factor with subsequent plant height. The relationship between initial height and final height by individual was also compared. The results suggest that there is a significant, but weak direct relationship between the two (R^2=0.44, p-value<0.001, Fig. A.3). This demonstrates that the indidivuals had a consisten growth pattern across treatments, where the larger the plant at the time of outplanting, the larger the same individual at the end of the 12 month period.

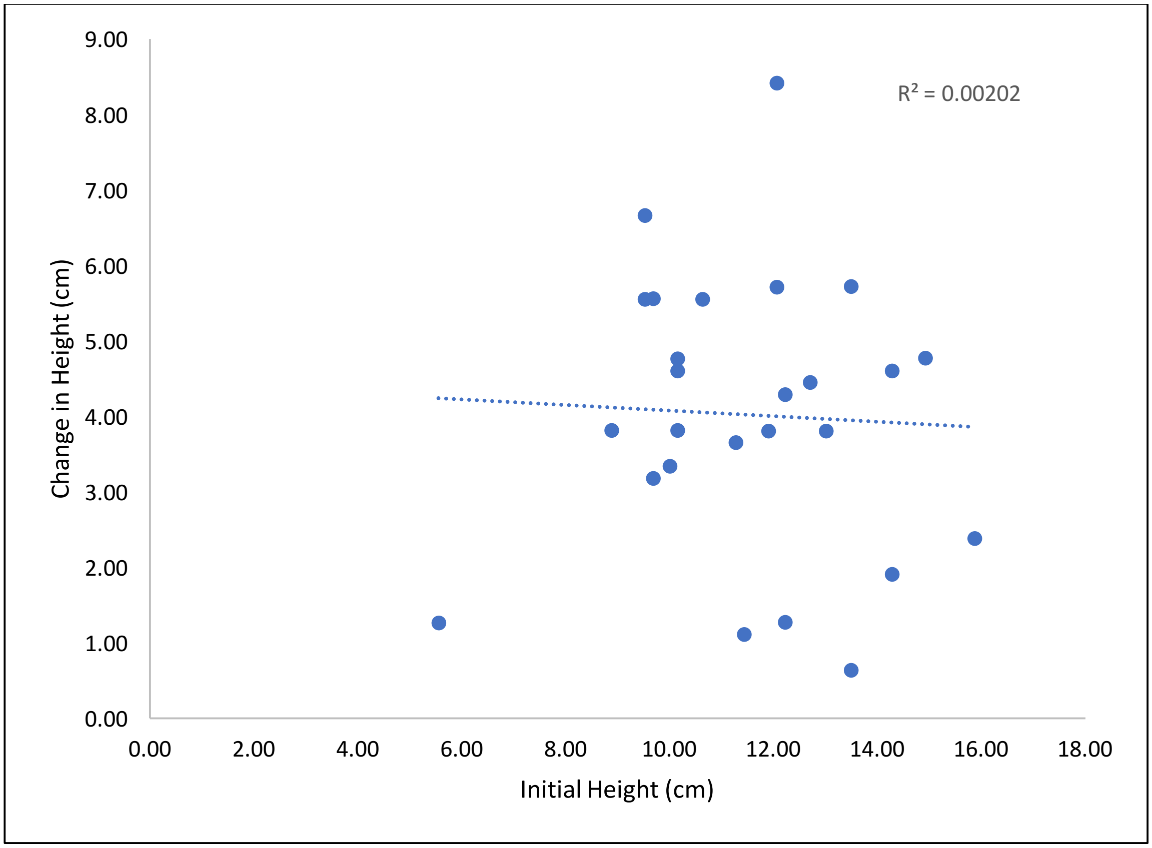

Figure A.2 Scatterplot showing the correlation of the absolute change in height with the initial height of *Cyrtandra kaulantha* individuals (beginning to end of the field study; treatment groups not relevant).

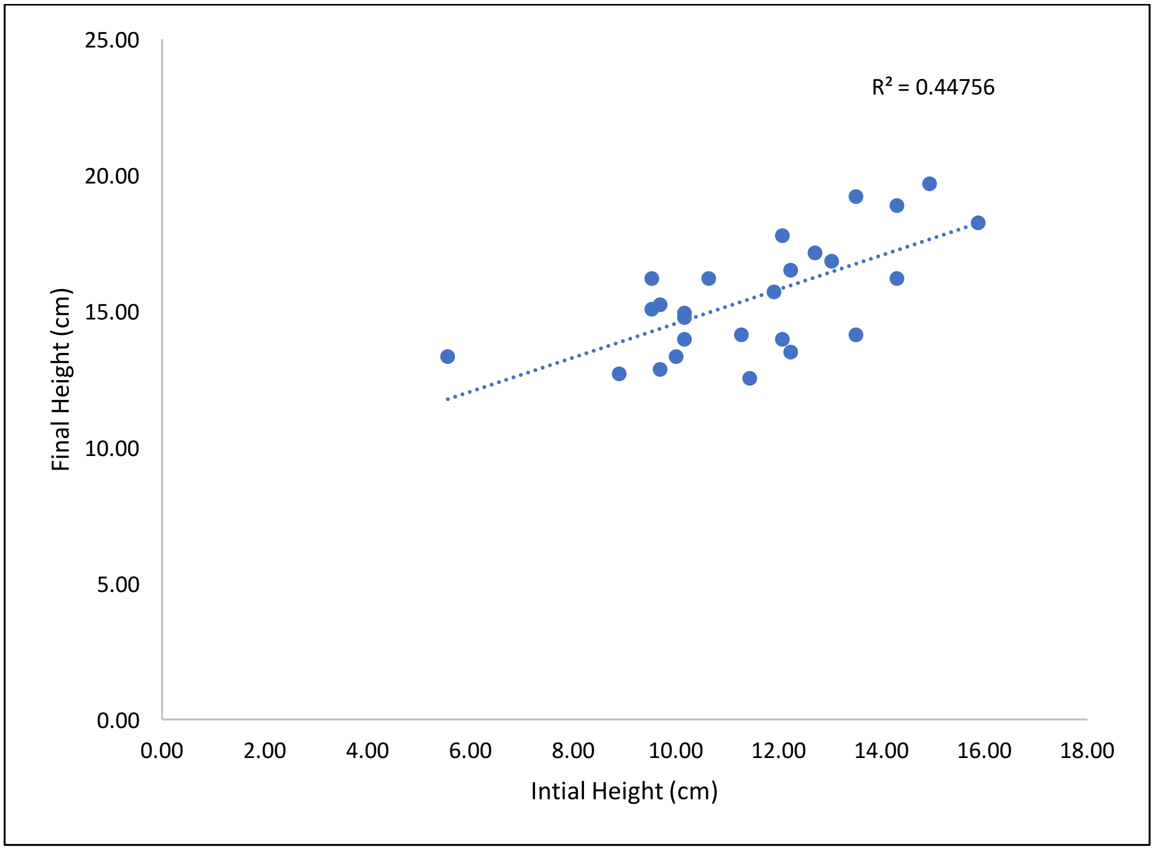

Figure A.3. Scatterplot of the correlation of Final Actual Plant Height (cm) by Initial Plant Height (cm)of *Cyrtandra kaulantha* individuals (beginning to end of the field study; treatment groups not relevant).

A.3 RELATIONSHIPS BETWEEN MORPHOLOGICAL MEASUREMENTS

The relationship between the each morphological measurement and stem height were compared through linear regression. The relationship between leaf area and height showed a weak but significant positive relationship (R^2=0.21, p-value<0.0001, A.4). This suggests that plant height and leaf area increase commensurately for this species. The relationship between basal diameter and stem height was not signifcant (R^2=0.0003, p-value=0.75, Fig. A.5), which indicates that basal *Cyrtandra kaulantha* does not grow uniformly and thatthe height of the plant had no measured effect on the diameter. The relationship between leaf number and plant height showed a very weak but significant negative relationship (R^2=0.135, p-value<0.0001, Fig. A.6).

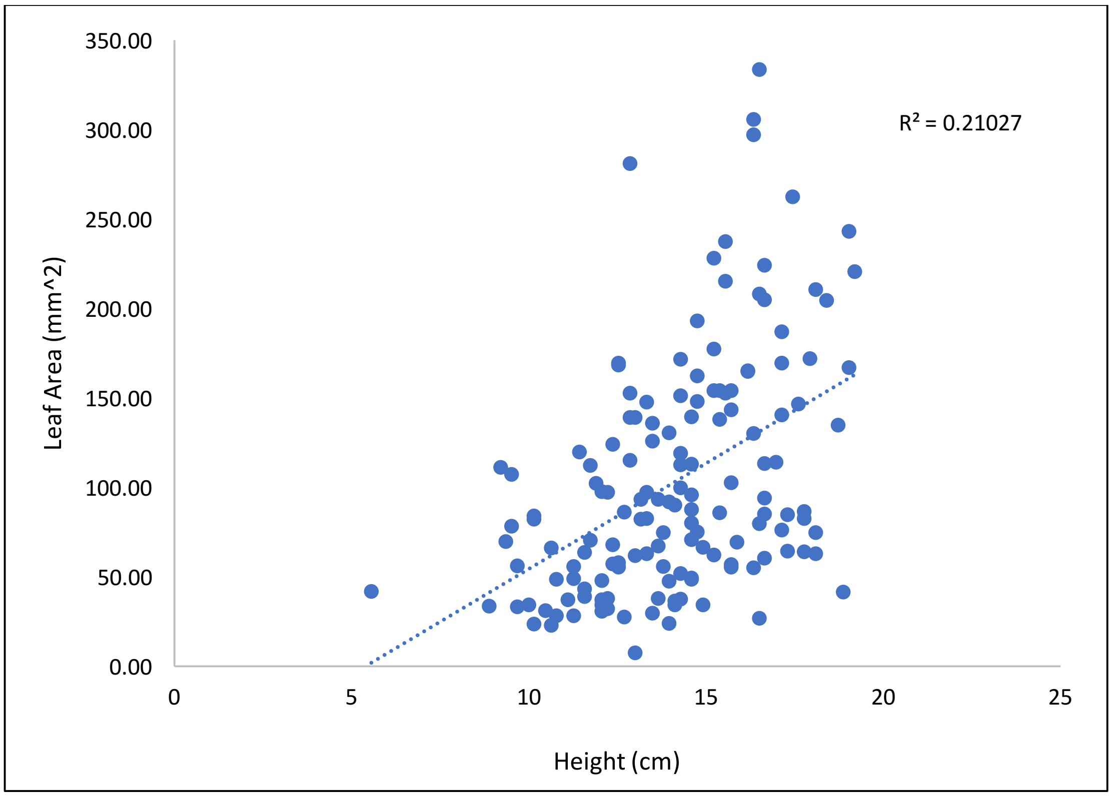

Figure A.4. Relationship between the measured plant height of *Cyrtandra kaulantha* individuals and the leaf area as measured by ImageJ; Treatment groups not considered

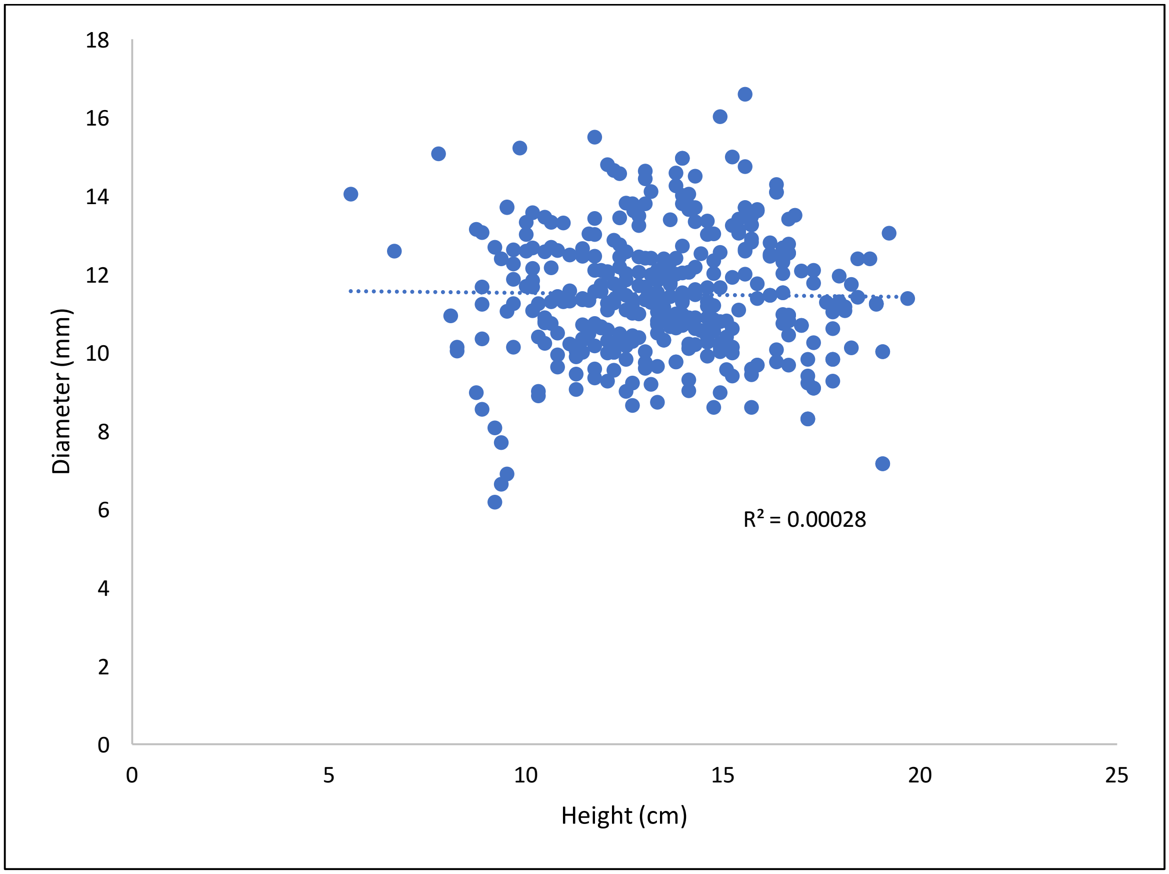

Figure A.5. Relationship between the measured plant height of *Cyrtandra kaulantha* individuals and the stem basal diameter as measured by caliper; Treatment groups not considered

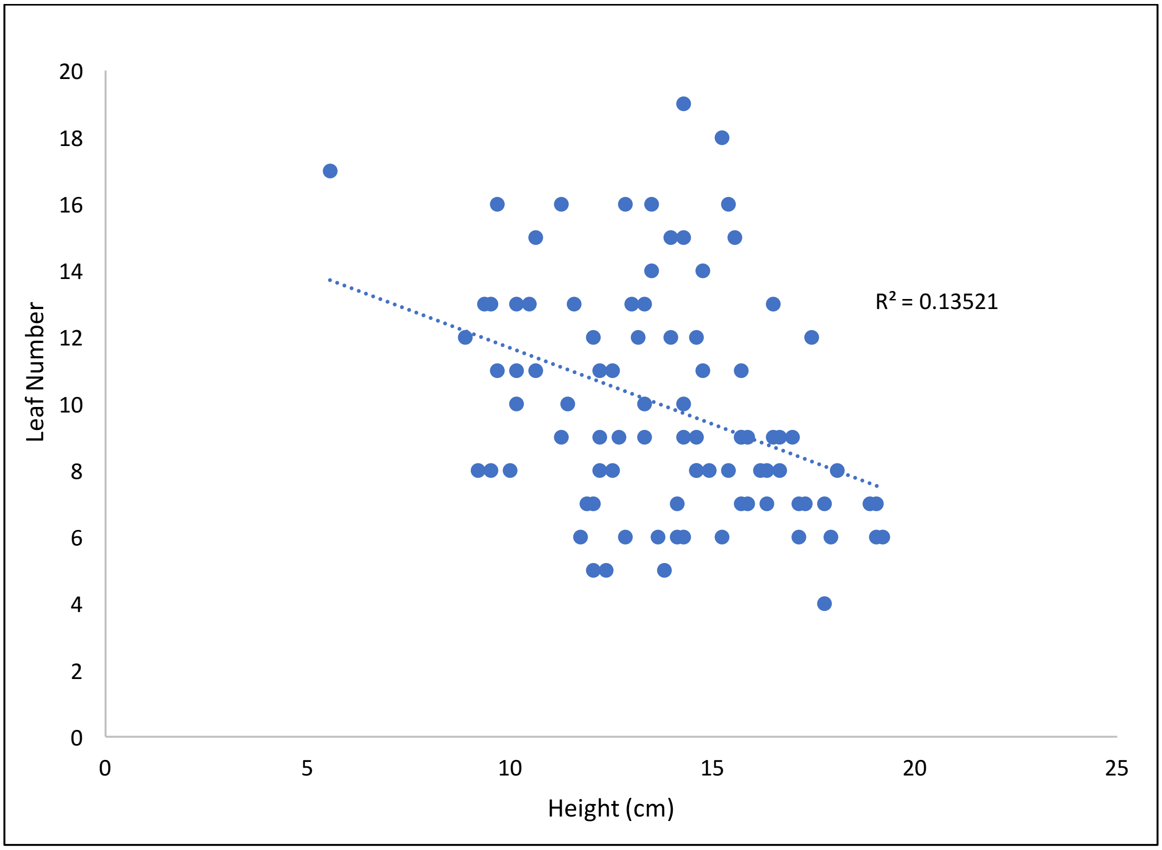

Figure A.6. Relationship between the measured plant height of *Cyrtandra kaulantha* individuals and the number of leaves per plant; Treatment groups not considered.

The relationship between leaf number and leaf area was compared to see if there was confounding correlation between similar morphological traits. The relationship between the two was not significant (R^2=0.004, p-value=0.56, Fig. A.7).

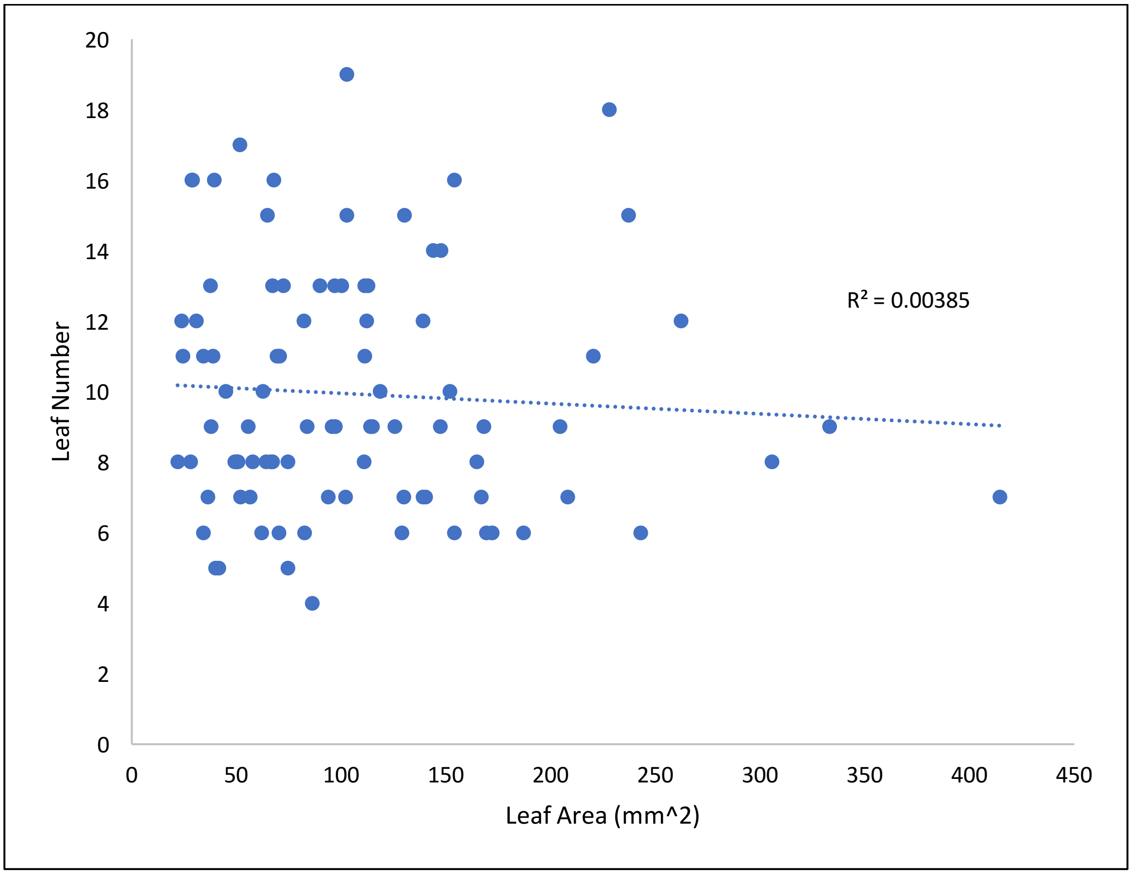

Figure A.7. Relationship between the measured leaf area of *Cyrtandra kaulantha* individuals and the number of leaves per plant; Treatment groups not considered

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